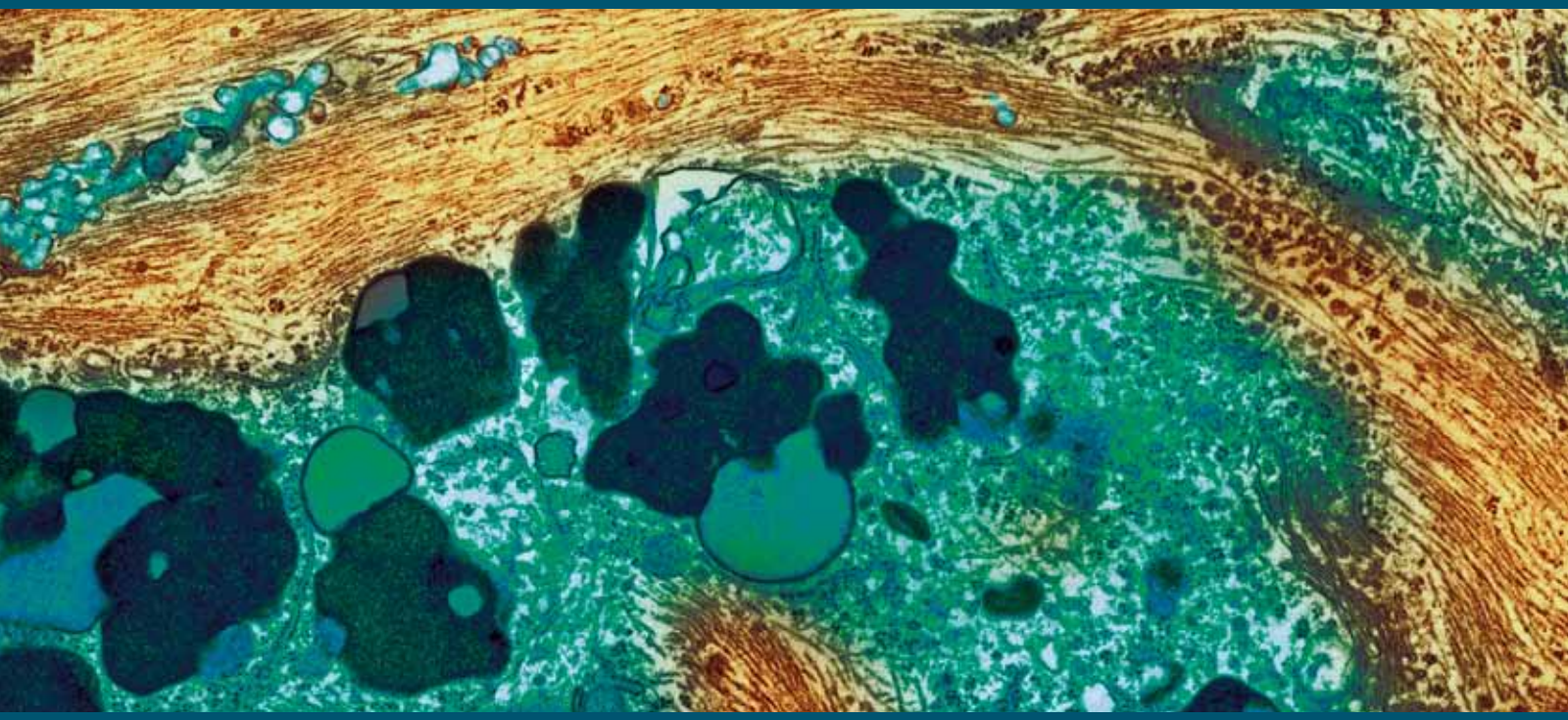
International Journal of Alzheimer's Disease

Tau Protein: Function and Pathology

Guest Editors: Hanna Rosenmann, David Blum, Rakez Kayed, and Lars M. Ittner



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Editorial Board

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Editorial

Tau Protein: Function and Pathology

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In the last decade, the fundamental role of the microtubule-associated protein tau in neurodegeneration and dementia has been widely accepted. The generation of various transgenic models for tau pathology, varying in expressed mutations and driving promoters with either permanent (constitutive) or inducible expression, as well as the use of alternative animal models (fly, zebra fish, and rat) provided tools for studying mechanistic aspects of tau pathology and developing therapeutic approaches. It is now well established that pathological forms of tau (hyperphosphorylated, aggregated, and truncated) are a major cause of dementia, rather than being only a secondary effect to the amyloid pathology in Alzheimer's disease (AD). There is a link between these two AD pathologies, tau and amyloid, with tau pathology being downstream to amyloid pathology, yet tau pathology can develop and respond independently of amyloid plaques. The direct evidence for tau pathology developing independently of amyloid, being sufficient to cause dementia and neurodegeneration, is the fact that there are various diseases with isolated tau pathology (frontotemporal dementia, Pick's disease, etc.), one disease with both pathologies (AD), while there is no dementia disease with isolated amyloid pathology without tau pathology present. These findings supported the concept that amyloid toxicity is tau dependent and that blocking/reducing the pathological effects of tau may be protective against the harmful effects of amyloid pathology, a concept that has indeed proven feasibility in various studies.

Much evidence has been accumulated pointing to the contribution of tau to AD pathology by two mechanisms: loss of function (such as stabilization of microtubules) as well as gain of toxic function (aggregation and deposition as neurofibrillary tangles). Recently, new concepts emerge contributing to our understanding of the pathogenesis of tau pathology, particularly the identification of toxic soluble oligomers of tau, arguing for these isoforms being the main toxic forms of the taupathology; and the concept that tau pathology may spread in the brain by a prion-like mechanism, possibly involving a transsynaptic mechanism of spreading along anatomically connected networks.

These accumulating data provide a better understanding of tau pathogenesis, and given the disappointing clinical outcomes of antiamyloid therapeutic approaches, led the scientific community to devote much more effort into studying tau pathology, and into developing tau-targeted therapeutic approaches, such as tau immunotherapy, kinase inhibitors, or microtubule stabilizers.

In this Special issue of International Journal of Alzheimer's Disease, the investigators contributed review articles as well as original research articles that stimulate the continuing efforts towards understanding tau pathology in AD and other tauopathies as well as unravel the physiological functions of tau, in an effort to develop new treatments.

The paper by the F. Van Leuven's group: "Protein tau: prime cause of synaptic and neuronal degeneration in Alzheimer's disease," which discusses the relevance of tau in Alzheimer's disease and frontotemporal dementias. This concise and clear review covers the major discoveries and the many still remaining questions in the field.

The L. M. Ittner group contributed an excellent review paper: "Lessons from tau-deficient mice." This paper takes you through the under-reported part of the tau story ; it summarizes the consequences for tau loss of function and its possible role in neurodegeneration. The authors thoroughly reviewed the different tau knockout models and addressed the pathophysiology of various tau models. Tau mediates Aβ toxicity and perhaps the toxicity of other amyloidogenic proteins, hence, the characterization of tau knockout animal models is critical for our understanding of the complex molecular signaling pathways in Alzheimer's Disease.

S. S. Hébert and his colleagues, review in their article: "MicroRNAs and the regulation of tau metabolism" what is known about the transcriptional and posttranscriptional regulation of tau. They discussed clearly the role of micro RNAs (miRNAs) in this process, with a focus on miR-16 and miR-132 as putative endogenous modulators of neuronal tau phosphorylation and tau exon 10 splicing, respectively. They speculated that miRNAs may contribute to sporadic forms of tauopathies.

The review from M. Gistenlinck and collaborators, entitled "*Drosophila* models of tauopathies: what have we learned?" is focused on how and why Drosophila is helpful to modelize Tau pathology. Further, contributors explain why such simple models are important to unravel new pathophysiological hypothesis from genetic screening. Finally, authors describe clearly how Drosophila models are unvaluable tools to reconcile genome wide association studies with pathophysiology.

The group of Gozes in the paper: "Tau and caspase 3 as targets for neuroprotection" shows clearly that in an in vitro model for ischemia, in primary neuronal cultures subjected to oxygen-glucose deprivation that causes an increase in the levels of active caspase-3 and hyperphosphorylated tau, both processes are prevented by either the NAP peptide or caspase-inhibitor treatment. This group suggests that caspase activation may be an upstream event to tau hyperphosphorylation.

M. Kolarova and colleagues, in their contribution entitled "Structure and pathology of tau protein in Alzheimer disease" give us a comprehensive overview of what tau is and how tau can be modified. This contribution particularly emphasized that posttranductional modifications of Tau are important regulatory steps of its aggregation. Also, authors point out importance of Tau truncation which may additionally play an important role in AD-related pathophysiology.

The final paper of this special issue entitled: "Tau phosphorylation by GSK3 in different conditions" J. Avila et al. review comprehensively the complex consequences for tau of being a phosphoprotein. Focusing on serine/threonine phosphorylation they discuss that depending on the modified residue in tau molecule, phosphorylation could be protective, like in processes like hibernation, or toxic like in development of tauopathies, characterized by a hyperphosphorylation and aggregation of tau.

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Review Article

Protein Tau: Prime Cause of Synaptic and Neuronal Degeneration in Alzheimer's Disease

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The microtubule-associated protein Tau (MAPT) is a major component of the pathogenesis of a wide variety of brain-damaging disorders, known as tauopathies. These include Alzheimer's disease (AD), denoted as secondary tauopathy because of the obligatory combination with amyloid pathology. In all tauopathies, protein Tau becomes aberrantly phosphorylated, adopts abnormal conformations, and aggregates into fibrils that eventually accumulate as threads in neuropil and as tangles in soma. The argyrophilic neurofibrillary threads and tangles, together denoted as NFT, provide the postmortem pathological diagnosis for all tauopathies. In AD, neurofibrillary threads and tangles (NFTs) are codiagnostic with amyloid depositions but their separated and combined contributions to clinical symptoms remain elusive. Importantly, NFTs are now considered a late event and not directly responsible for early synaptic dysfunctions. Conversely, the biochemical and pathological timeline is not exactly known in human tauopathy, but experimental models point to smaller Tau-aggregates, termed oligomers or multimers, as synaptotoxic in early stages. The challenge is to molecularly define these Tau-isoforms that cause early cognitive and synaptic impairments. Here, we discuss relevant studies and data obtained in our mono- and bigenic validated preclinical models, with the perspective of Tau as a therapeutic target.

1. Introduction

It is no coincidence to begin this paper on protein Tau and tauopathies by referring to Alzheimer's disease (AD), because it is the most frequent tauopathy, albeit a "secondary" one. More than hundred years after the first documented case of AD, the etiology of sporadic forms remains hitherto unknown. Demographic changes, increased life expectancy with a fast growing elderly population, lead us and all future generations to the dramatic increase in the incidence and prevalence of AD—described by some as "the pandemic of the 21st century" [1].

The extracellular amyloid deposits, c.q. neuritic plaques and cerebrovascular angiopathy, together with the intracellular neurofibrillary threads and tangles (NFT) remain the key pathological markers in AD brain. Nevertheless, the essential basis of cognitive impairment is generally accepted to be reduced synaptic plasticity, evolving into loss of synapses and eventually loss of neurons only in later stages [2–5]. Extensive genetic and biochemical data have implicated the amyloid peptides, the main components of the amyloid deposits, as the central mediators of AD. The amyloid cascade hypothesis, prevailing for more than two decades, stated that the amyloid deposition is the initiating event of neuronal dysfunction and cell death in brain [6]. In familial AD-cases, production of more and longer amyloid peptides, for example, Aβ1–42, by abnormal proteolysis of the membrane-bound amyloid precursor protein (APP) triggers a pathologic cascade, years, or decades before the overt clinical manifestations of the disorder [7–9]. By extension, a similar pathogenic pattern is proposed in sporadic AD cases, whereby increased levels of similar or other amyloid peptides, for example, Aβ1–38, Aβ1–43, pE3Aβ3-x among others, can stem from deviating proteolysis, increased production, failing degradation, and ApoE-mediated elimination from the brain.

The major—if not only—arguments that support the amyloid hypothesis are genetic by nature [12]. The detection

of different mutations in the gene coding for APP, but even more so in the gene coding for presenilin-1 (PS1) is defined as causing early onset familial AD [12]. Conversely, overproduction of amyloid peptides from mutant APP, alone or in combination with mutant PS1 in transgenic mouse brain, failed to cause appreciable tauopathy or neuronal degeneration. The question arose whether accumulating amyloid peptides are indeed the real culprit for neurodegeneration in AD. Recent neuroimaging studies demonstrated considerable amyloid deposits in the brain of a sizeable fraction, that is, 15–30% depending on the study, of cognitively normal individuals, whereas clinically diagnosed AD patients can show no amyloid deposition by PET scan [13, 14]. These and other observations have led to the modified amyloid hypothesis, stating that not deposits but soluble toxic amyloid species must initiate AD-pathology, and moreover, that tauopathy is essentially implicated [15].

For decades, the microtubule-associated protein Tau was known as the main component of NFT, and although occupying the number two spot on the list of proteins implicated in AD, the focus of the field is traditionally aimed on amyloid. Long-standing observations, nevertheless, indicate that neuronal tauopathy provides the closest pathological approximation of clinical defects observed during the life of AD patients. The typical brain-regional occurrence and progression of Tau pathology correlates temporally and spatially well with neuronal and cognitive dysfunction [16, 17], with the added weight of the correlation to CSF-levels of phosphorylated Tau [18]. Therefore, the crucial question remains where protein Tau should be placed in the amyloid cascade hypothesis: downstream, at the same level or even upstream of amyloid? Should we consider protein Tau as a by-stander of the amyloid toxicity, as a mediator or even as a prime player, and therefore preferred target? Only recently fundamental scientist have gained more insight into how these two main defects and players could be linked mechanistically.

Important is that even in young familial AD-cases, amyloid pathology is always accompanied by Tau pathology, similar to late sporadic cases of AD. This establishes tauopathy even more firmly as cause, besides or rather than codiagnostic hallmark in all AD cases! Scientific reasoning takes this one step further, to implicate tauopathy in the more early stages of AD, conform to the origin of pathology in primary tauopathies. Important caveat here is that the early stages of Tau pathology are yet to be defined molecularly in human patients. This was and remains a formidable scientific challenge: biochemical and biophysical definition of the neurotoxic Tau species, which we have termed Tau-P* [19].

2. Protein Tau: Physiology and Pathology, an Unstable Balance

In contrast to the apparent redundancy of the physiological function of protein Tau, as demonstrated in Tau-deficient mice [20], the presumed pathological importance of protein Tau was established by the discovery of inherited mutations in the MAPT gene. These are tightly linked to the disease in families suffering autosomal dominant frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) [21–24]. Once more, genetic findings established a key factor in the pathogenesis of a disease, in this case protein Tau in a subgroup of FTD, a heterogeneous group of tauopathies characterized by dementia and movement disorders.

The structure of the Tau gene has been reviewed in detail elsewhere [25, 26]. Alternative mRNA splicing of exon 10, encoding the second of four microtubule-binding domains (MTBD) produces two major isoforms, denoted as Tau.3R and Tau.4R, respectively, with lower and higher affinity for microtubules (MT) [27–29]. The abundance of Tau.3R and Tau.4R in human brain changes during brain development and neuronal differentiation. In embryonic brain, Tau.3R isoforms dominate, apparently providing structural and morphological plasticity to developing and differentiating neurons. In mature neurons, Tau.4R gains importance and becomes largely located in axons, excluded from soma and dendrites where other MAPs predominate. In the adult human brain, Tau.3R and Tau.4R isoforms are balanced, while in adult mouse brain the Tau.4R isoforms prevail [30, 31].

Importantly, both intronic and exonic mutations in the MAPT gene are associated with FTDP-17. Many are located in exon 10 or in the surrounding introns, affecting the alternative splicing of this exon. Evidently, intronic mutations produce normal, wild-type proteins, which in case of protein Tau result in the distortion of the normal isoform-balance. An abnormal Tau.3R/4R balance, either increased or decreased, is thought to disturb the function of Tau in MT assembly, stabilization, and transport. Expressed mutations then are presumed to provoke similar problems, while mutant Tau in general is more prone to phosphorylation and polymerization [27–29].

Immunohistochemical and biochemical studies, supported by genetic data, have revealed that in specified tauopathies the pathology directly reflects the aberrant Tau isoform ratio. For instance, in PSP, CBD, and AGD, the inclusions contain mainly Tau.4R isoforms [30, 32, 33], whereas Pick bodies contain mostly Tau.3R. Furthermore, in familial FTD, exonic mutations, for example, P301L, P301S, G272V, N279K, V337M, R406, cause significant overrepresentation of Tau4R [34, 35]. In AD and other tauopathies, variable admixtures of the two major Tau isoforms are biochemically detected in aggregates and deposits in soma, dendrites, and axons. Obviously, an inconsistent picture emerges of Tau isoforms biochemically associated with different tauopathies, although all are pathologically defined by argyrophilic tauopathy.

Our eventual molecular understanding of the role played by abnormal structural features of protein Tau in human tauopathies must ultimately be based on the knowledge of its normal cellular functions. The major, and as far as we know, only physiological function of protein Tau involves its capacity to bind to microtubules to control or affect spacing, stabilization and dynamics [36–38]. That protein Tau is involved in signal transduction, neuronal differentiation, organellogenesis and growth can likely be reduced to the same primary act of MT-binding [39–41]. We will not indulge in describing

the processes in which protein Tau has been implicated or proposed, a subject reviewed recently elsewhere [42].

The function of Tau in adult—and ageing—brain is supposed to be fine-tuned by post-translational mechanisms besides the alternative mRNA splicing producing Tau.3R and Tau.4R isoforms. The prime one is phosphorylation of a limited set of serine and threonine residues, selected among the 79 available. With respect to the mechanism governing this selection, one must not only consider the specificity of different kinases responsible, but also the naturally unfolded nature of protein tau, which presumably exposes most, if not all the S/T residues.

3. Phosphorylation, Missorting, and Aggregation: In What Order and Extent?

Over the last years, following and thanks to the genetic discoveries in the MAPT gene, striking observations in patients but mainly in experimental models have changed some long-established convictions concerning the role of protein Tau in cognitive disorders and neurodegeneration.

In transgenic mice, regulated expression and suppression of mutant protein Tau correlated with defective and improved cognition [43]. Moreover, protein Tau but not NFT appeared involved in neuronal death in transgenic mice and AD patients [44, 45]. Elimination of protein Tau in an amyloid mouse model restored cognitive and behavioral deficits [46], corroborating observations in AD patients that tauopathy defines the cognitive demise, discussed above. Moreover, not APP but protein Tau proved neurotoxic for pyramidal neurons in CA1 and cortex in a nontransgenic, AAV-based model, with formation of oligomeric but not fibrillar Tau [47].

Ideas that have been simmering for years are more and more substantiating that specified neuronal dysfunctions are evident before NFTs are deposited. Moreover, the synapto- and neurotoxicity of protein Tau depends on its posttranslational modifications, in first instance O-phosphorylation, but also others that need to be explored in detail: Y-phosphorylation, glycation, glycosylation, acetylation, sumoylation, among others [48–52]. Therefore, accurate temporal and spatial patterns of pathological traits that must also be physiologically important deserve investigation to understand their role in the genesis and evolution of AD and tauopathies.

In general, increased phosphorylation of protein Tau decreases its binding to MT and thereby regulates the normal biological activity of protein Tau related to microtubule spacing, assembly and stability. Obviously, phosphorylation of protein Tau is part of normal physiological regulatory processes and not pathogenic per se [39, 53]. Conversely, in AD and all primary tauopathies, protein Tau becomes more and more phosphorylated with disease progression, to enter a state that is generally referred to as "hyper-phosphorylated." Nevertheless, no exact lower or upper limits are defined to this connotation, which exposes a major molecular and analytical problem: how many of the vast number of potential O-phosphorylation sites in protein Tau are physiologically relevant? In second order, the question is, are these sites directly pathologically implicated—or merely correlated? We currently address these questions by "timeline" analysis of transgenic mouse brain to define which phosphorylation levels and sites generate or accelerate disease.

While several studies suggest that the functional impact of phosphorylation depends on the specific phosphorylation sites [54–56], others support the notion that overall increased, not site-specific, phosphorylation is sufficient [57]. It is well-known that in development and postnatally, protein Tau is heavily phosphorylated. This "fetal Tau" binds less to MT and helps maintain dynamics of microtubule assembly/disassembly during this period of most active neurite outgrowth, albeit without eliciting tauopathy or neurotoxicity as in ageing brain. Differentiating functional differences between normal Tau in the immature brain and pathological Tau in ageing brain might define phosphorylation of Tau at some specific sites, for example, S199, T231, and S396 in AD brain [58].

Tau phosphorylation is markedly increased in response to various stressors. Phosphorylation, although to lesser levels than in AD brain, appears to be mobilized by neurons to regulate activity of Tau transiently and reversibly as required. A prime example is hibernation as adaptive process that represents a powerful physiological strategy to withstand periods of limited energy supply. Hibernation is a hypometabolic state with declining body temperature, periodically interrupted by brief spontaneous periods of rewarming to core temperatures, whereby selective expression and control of kinases and phosphatases is an adaptive response for long-term survival [59]. Highly phosphorylated protein Tau readily accumulates and even appears to aggregate in brain of hibernating animals, while most interestingly the entire process is fully reversed when animals arouse, restoring normal temperature and metabolism without harming neurons or networks [60–62].

Other conditions, associated with reduced body temperature and increased Tau phosphorylation are starvation [63], cold water stress [64], and anesthesia [65]. Several reports suggest that anesthesia-induced hypothermia increases the risk of AD [66, 67]. Temperature fluctuations affect the relative balance of kinase and phosphatase activity [65, 68]. In a mouse model with incipient neurofibrillary pathology, the pool of free hyperphosphorylated Tau was recruited into Tau aggregates that accumulate over time [69]. Moreover, isoflurane anesthesia in young Tau.P301L transgenic mice provokes brainstem tauopathy and upper airway defects, suggesting similar problems in elderly patients exposed to anesthetics for surgery [70].

4. Tau Kinases: GSK3 Accepted, Others Explored...

The GSK3 kinases appeal as candidates to modulate Tau phosphorylation for several reasons: (i) they are abundantly expressed in neurons, (ii) their levels and/or enzymatic activity is increased in AD brain [71, 72], and (iii) they can phosphorylate many of the S/T-P sites of protein Tau that are

also phosphorylated in AD brain, that is, S199, T231, and S396 [73–75]. Additionally, in our transgenic Tau mouse models, GSK3 greatly increased the severity of tauopathy when expressed in conjunction with mutant Tau.P301L [76, 77], but alleviated the axonopathy when coexpressed with wild-type Tau.4R [78]. Moreover, and surprising for a naturally unfolded protein is the notion that phosphorylation affects the conformation of protein Tau. Monomeric Tau in solution, when phosphorylated at critical sites either upstream or downstream of the MTBD, appeared to adopt a more compact conformation that was proposed to reflect its propensity to aggregate [79].

The long-standing definition of protein Tau as an axonal protein, and even axonal marker [80, 81], contrasts with the early pathological defects observed in most tauopathies: abnormal delocalization into the somatodendritic compartment where protein Tau becomes even more phosphorylated and subsequently forms the well-known aggregates and NFT. Several hypotheses for the axon-specific location were proposed: axon-specific sorting of mRNA coding for protein Tau or preferential degradation of mRNA and/or protein Tau in dendrites, affected or even steered by selective phosphorylation of protein Tau [82–84]. In the last years, among efforts to clarify these issues a new proposal for a retrograde filtering action dependent on microtubules would allow protein Tau to move into but not out of axons [85]. Moreover, once protein Tau becomes phosphorylated, its microtubular interaction is decreased or disrupted, and phospho-Tau can bypass that barrier. A different mechanism depends on the activation of the PAR-1/MARK kinase to phosphorylate protein Tau downstream of signaling initiated by amyloid [86], thereby contributing to relocate protein Tau. This would explain its effects on synaptic trafficking, anchoring of glutamate receptors, and interaction with other kinases in disturbing postsynaptic functions [40, 87].

5. The Neurotoxic Species, Known Only as Tau-P*

Intracellular aggregation and deposition of protein Tau as NFT in soma and processes is commonly observed in all tauopathies, including AD. These aggregates, predominantly composed of highly phosphorylated protein Tau, are detergent insoluble and presented either as paired helical filaments (PHF), twisted ribbons, or straight filaments, usually in varying combinations. Although the load of NFT correlates with the severity of cognitive impairment in humans, mouse models for tauopathy demonstrate memory defects to precede NFT-like tauopathy [19, 77, 88, 89]. Moreover, in a conditional model, reversal of Tau expression reversed the cognitive defects even without affecting the Tau deposits [43, 90]. The levels of early multimeric Tau-aggregates that preceded the NFT were found to correlate with memory deficits [91]. These findings corroborate the hypothesis that molecularly unspecified intermediates in the Tau-aggregation cascade, situated between single Tau molecules and large fibrils, are the actual neurotoxic agents. The connotation Tau-P*, as defined previously [19, 92], represents intermediate forms of phosphorylated protein Tau, which remain to be defined molecularly. They are proposed to adopt a transitional conformation state and to be the effective executers of synaptic and eventually neuronal toxicity, if or when they are not directed into formation of the large fibrillar Tau-aggregates that we now consider not or less harmful for neurons. Therefore, decoding the sequence of events that transform soluble or MT-bound Tau into a toxic molecular intermediate that only later becomes an inert aggregate is the key to understand ontogenesis and evolution of any tauopathy. In addition, these studies entertain the idea that therapeutic intervention is possible by acting on the expression, phosphorylation, and aggregation of protein Tau.

The formation of NFT involves sequential steps as studied best with recombinant proteins *in vitro* and, because cellular models are still lacking, are corroborated by the timeline of events in transgenic mouse brain *in vivo* [93]. The first step is believed to be the neutralization of the protein Tau-microtubule interaction by phosphorylation by different kinases, increasing the cytoplasmic levels of unbound protein Tau. Moreover, while protein Tau lacks a well-defined structure, the initial phosphorylation promotes an unspecified molecular state that appears favorable for dimerization and aggregation [79, 94]. The adopted presumed β-sheet structure involves the MTBD of protein Tau, whereby eventual disulfide bridging, although not generally accepted, could help shift the equilibrium from soluble monomers to higher-order multimers [95, 96]. Subsequently, further association of β-fibrillar Tau-multimers leads to filaments that deposit as neuropil threads and tangles in the soma.

Biochemical indices for the occurrence of oligomers were interpreted to represent off-pathway aggregates that do not form filaments [97]. Whether or not hyperphosphorylation of protein Tau is an indispensable event for the initial dimerization remains elusive and debated. *In vitro* studies demonstrated that fibrillar assemblies of protein Tau are not per se phosphorylation dependent [74] and even that the propensity of protein Tau to form multimers is reduced by phosphorylation particularly of S262 by MARK2/Par1 [98]. Moreover, even the inverse order of events was demonstrated in a mouse model for aggregation of protein Tau, because the incipient aggregation was the actual trigger for subsequent phosphorylation of protein Tau [99]. Consequently, defining the molecular identity of the intermediate Tau species referred to as Tau-P*, a hyperphosphorylated, conformational small aggregate, will be a major step forward on the arduous route to the most appropriate therapeutic target in AD, and in primary tauopathies.

6. Brain-Regional Propagation of Tauopathy

Anatomical patterns of the pathology in human brain suggest, but do not prove, a progressive spreading of tauopathy with progressing cognitive decline [100]. Initial depositions of phosphorylated protein Tau are first observed in the locus coeruleus, one of several subcortical nonthalamic nuclei that has diffuse projection to the cortex. Subsequently, immunochemical defined phosphorylated protein Tau is observed in entorhinal cortex and in its efferent hippocampal and neocortical regions [101].

This apparent spreading of the tauopathy throughout human brain in a specified regional pattern has been the subject of various hypotheses. Initial evidence for cell-to-cell transfer of protein Tau aggregates came from two studies carried out in cells and *in vivo*. The first demonstrated that fibrils of protein Tau added to the culture medium were taken up by neuronal cells and caused formation of Tau-aggregates in the cytoplasm [102]. The second reported that in mouse models of tauopathy, intracerebral injection of brain tissue extracts containing Tau-aggregates initiated spreading of intracellular Tau-aggregation from the injection site to other brain areas, although not producing neurodegeneration [103]. These experimental studies still remain controversial and need to be confirmed, eventually to define the route and mechanism whereby cytoplasmic aggregates of protein Tau can become secreted and taken up by neurons, in order to establish the transmittance between neurons as a potential mechanism of spreading of tauopathy in human brain [104]. Most recent data from novel mouse models that express Tau.P301L specifically and only in the entorhinal cortex (ERC) corroborate the hypothesis of cell-to-cell spreading of tauopathy, but leaves the responsible mechanisms open for speculation [105, 106].

7. Mouse Models for Tau Pathology

To understand the impact of Tau phosphorylation and aggregation in synaptic and neuronal degeneration, fully characterized mouse models that exhibit the typical phenotypic features and pathological changes of human tauopathies are needed. Many transgenic mouse models for tauopathy have been developed and characterized over the last decade and were the subject of some recent reviews [19, 88, 107]. Here, we limit the discussion to our mouse models that represent or recapitulate interesting aspects of the pathology of protein Tau in Tau pathologies, including AD.

7.1. Tau.4R Mice. They express full length, wild-type human Tau under control of the mouse Thy-1 gene promoter in the FVB/N genetic background. The advantage of the mouse Thy-1 gene promoter permits neuron-specific expression of the transgene, which begins in the second week postnatally, avoiding interference with development. Tau.4R mice present increased phosphorylation of human protein Tau, however without formation of any Tau-aggregates even at late age [108]. Conversely, Tau.4R mice develop axonopathy, initially consisting first of axonal dilatations or spheroids, evolving into Wallerian degeneration with muscle wasting. The evident motor problems, already present at young age (6 weeks) in homozygous Tau.4R mice, are explained by excessive binding of protein Tau.4R to microtubules. This prevents the normal binding and passage of motor proteins responsible for axonal transport in both directions, resulting in the stochastic accumulation of transported items, from synaptic vesicles to mitochondria over Tau and neurofilament aggregates [108].

Remarkably, the severe axonopathy and motor problems hardly affect the survival of the Tau.4R mice [89, 108]. Moreover, the pathological phenotype was rescued by coexpression of GSK3β, resulting in bigenic mice that appear largely normal [78]. Molecularly, this is explained by the evident increased phosphorylation by GSK3β of human protein Tau.4R, which displaces it from the microtubules and thereby restores normal axonal transport in both directions. Despite the fact that protein Tau.4R became highly phosphorylated in brain of the Tau.4RxGSK3β bigenic mice, no Tau pathology resulted. Besides demonstrating for the first time *in vivo* that GSK3β is an effective Tau-kinase, the data also underlined that additional phosphorylation by other kinases is needed to develop tauopathy. A final argument explaining the lack of tauopathy in Tau.4R mice, and by extension in the bigenic Tau.4RxGSK3β mice, was our most recent finding of the strict axonal location of Tau.4R, as opposed to the somatodendritic delocalization of Tau.P301L in transgenic mouse brain [92, 109].

7.2. Tau.P301L Mice. In contrast to Tau.4R mice they exhibit less extensive phosphorylation of mutant protein Tau at younger age, but nevertheless develop into a representative model of tauopathy with neurofibrillary tangles and neuropil threads at older ages. These mice express the well-known human mutant Tau.P301L, the first to be associated with FTDP-17 [22, 23]. Human Tau.P301L is homozygously expressed under control of the same mouse Thy-1 gene promoter and in the same FvB/N genetic background as the Tau.4R mice [89].

Phosphorylation of Tau.P301L in mouse brain is initially low even up to the age of 7 months at the epitopes defined by Mabs AT8 and AT180, although phosphorylation is detectable with probes AD2 and AT270 [89]. The disease associated epitope AT100 was even completely absent at young age. In older Tau.P301L mice, phosphorylation of Tau increases leading to reduced affinity of the MT-binding and consequent delocation to soma and dendrites. The local aggregation into tangles and neuropil threads in terminal mice is demonstrated immunohistochemically with Mab AT100 and others, for example, MC1 and PHF1, and biochemically by hyperphosphorylated Tau in the sarkosyl-insoluble fraction (Figure 1) [89]. The development of the tauopathy becomes associated with motor problems, illustrated by clasping and rotarod performance even as early as age 7.5 months, because the hindbrain and particularly specified nuclei in the brainstem, for example, Koelliker-Fuse and raphe, are hit by the tauopathy.

Most recently, we discovered the associated reduction of involuntary control of breathing, resulting in upper-airway defects that cause excessive workload of the diaphragm, leading to impaired ultrasonic vocalization, exhaustion, and asphyxia, most likely associated with dysphagia in old mice. These defects then explain the clinical moribund phenotype of terminal Tau.P301L mice, with dramatic reductions in bodyweight: males drop below 20–18 grams, females below 16 grams. Premature death occurs mainly in the time-window of 8–10 months with the mean around age 9.4 months, not dependent on gender, and with practically no survivors beyond age 12 months. The terminal stage evolves

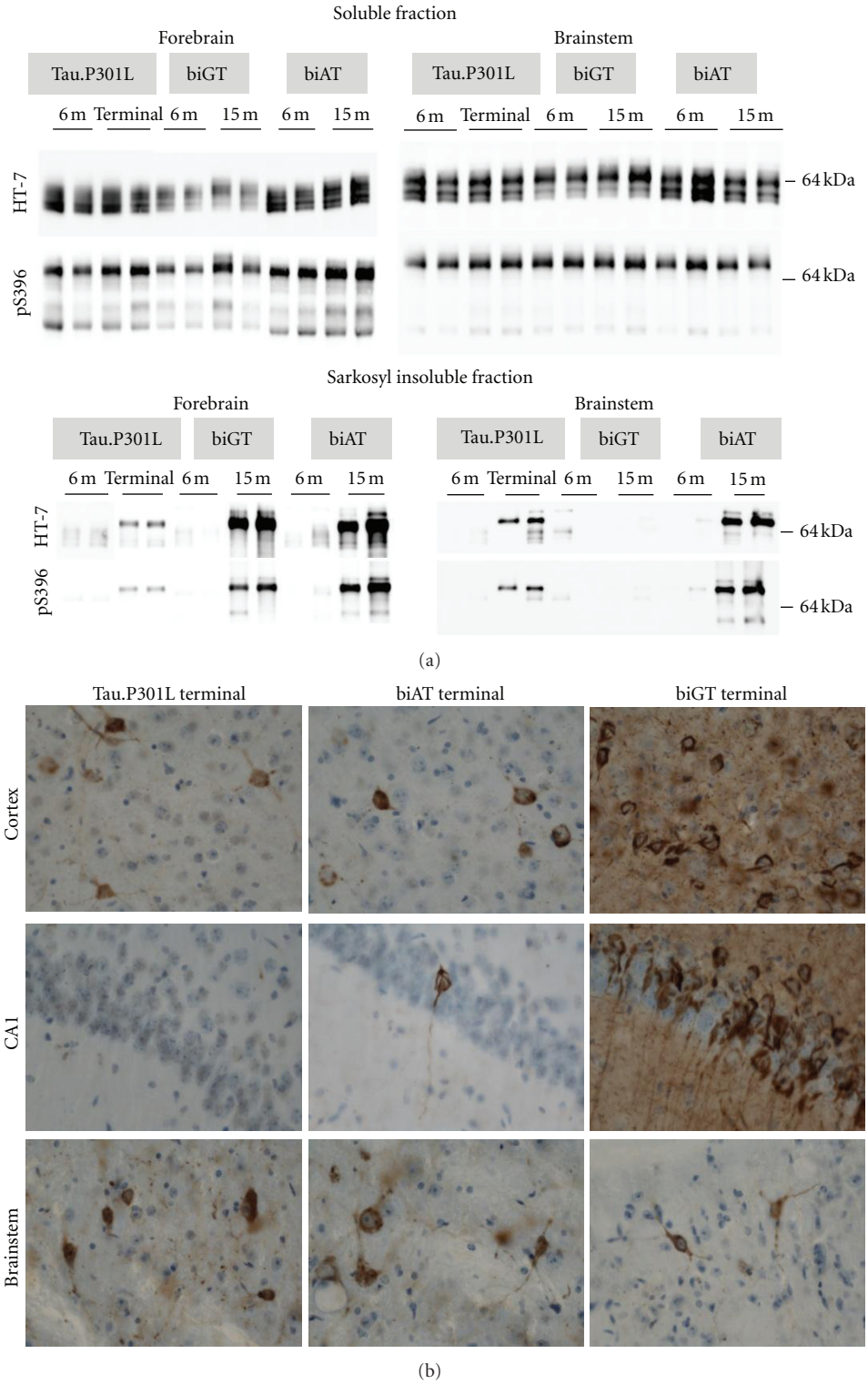

Figure 1: Selected biochemical and pathological characteristics of transgenic mouse models. (a) Soluble and sarkosyl insoluble fractions of Tau (SInT) were isolated as described [10] and proteins separated by SDS-PAGE on 10% tris-glycine gels. After transfer to nitrocellulose membranes, proteins were immunoblotted with either anti-pS396 (Invitrogen, Carlsbad, CA) or HT7 (Innogenetics, Gent, Belgium). Phosphorylated human Tau (apparent Mr about 64 kDa) is evident in soluble fractions from forebrain and brainstem of all three genotypes. SInT is evident in forebrain and brainstem of terminal TPLH and old biAT mice (age 15 months) but not in the brainstem of old biGT mice (15 months). (b) Immunohistochemistry with AT100 on free-floating sagittal sections of terminal mice shows tangles and neuropil threads in cortex and brainstem of all genotypes, but significantly less in the brainstem of biGT mice. Tauopathy is minimal or absent in pyramidal neurons of the hippocampus of terminal Tau.P301L transgenic mice.

aggressively and takes less than 2-3 weeks after the first signs of motor impairment, whereby the mice rapidly lose bodyweight, display a dystonic posture with progressive paralysis of all limbs and associated breathing problems leading to asphyxia, the presumed cause of premature death [11, 89, 110, 111].

Unexpectedly, the cognition of young Tau.P301L mice was markedly better than that of wild-type mice, with ameliorated long-term potentiation in the dentate gyrus and improved cognitive performance in object recognition tests [112]. This was anatomically substantiated by higher levels of mature spines in hippocampus and cortex compared to wild-type mice [109]. Although the spine maturation ratio remained high in hippocampus of adult Tau.P301L mice, the spines regress in length paralleling the impaired cognition, the increased phosphorylation and relocation of protein Tau.P301L from axons to soma and neuritic processes [109, 112].

Tau.P301L mice do not suffer any amyloid pathology and do not stand as a model for AD pathology, but these monogenic mice are an excellent model to study the impact of hyperphosphorylation and aggregation of protein Tau in different Tau pathologies. The progressive impairment that we documented in the Tau.P301L mice, starting from defective learning and memory in adults to problems at later age with breathing and vocalization, swallowing, movement, and motor defects, Tau phosphorylation, and aggregation into tangles, all correspond with features known from human tauopathies and neurodegenerative diseases. Needless to state that the effect of drugs or disease modifying treatments that target the pathological Tau, or its downstream partners or sequellae, can be evaluated in the monogenic Tau.P301L mice [19, 113].

7.3. biAT and biGT Mice. To define the relations and synergism between amyloid and Tau pathology in AD, and of the role of the GSK3 kinases, double transgenic mice were developed by combining homozygous Tau.P301L mice with either heterozygous human GSK3β.[S9A] mice or APP.V717I mice [77, 78, 114, 115], resulting, respectively, in GSK3βxTau.P301L (biGT) and APP.V717IxTau.P301L (biAT) bigenic mice. In both strains, the Tau pathology is aggravated with ageing by progressively increasing phosphorylation of Tau.P301L at typical pathological and conformational epitopes, resulting in the formation of highly fibrillary tangles and threads in cortex and hippocampus [77]. Conversely, in the brainstem of biGT mice the Tau pathology was significantly reduced relative to that in the parental Tau.P301L mice, which correlates with the markedly prolonged survival of the biGT mice compared to Tau.P301L mice: IC_{50} of 13 months versus 9.3 months, respectively.

Originally, we observed and described prolonged survival of the biAT mice, which was explained, by the increased activation of both GSK3 isozymes, that is, increased tyrosine phosphorylation, apparently driven by the high amyloid burden, as it was observed in the parental monogenic APP.V717I mice [77]. The subsequent in-depth further characterization of the biAT mice revealed a more complex pattern of mortality, which will be reported in detail elsewhere [116]. In brief, young biAT mice are prone to spontaneous or evoked epileptic seizures that cause a significant premature death in the tile-window of 1–6 months. Mortality is thereby evident at younger age and more frequent than in the parental APP.V717I mice [117].

Thereafter, mortality subsides, with a sizable fraction of biAT mice, particularly females surviving longer than the parental Tau.P301L mice. Of note, the female survivors develop, more severely than males, the typical combined AD-related pathology in relevant brain regions, that is, hippocampus, neocortex, entorhinal cortex, piriform cortex, and amygdala [77, 115]. In the forebrain of surviving biAT mice, the amyloid pathology with intracellular vesicular amyloid, extracellular diffuse and senile plaques, and vascular amyloid sets in at age from 10 to 12 months, preceding the Tau pathology. Of note, intracellular vesicular amyloid accumulation is already evident at young age (4–6 months) and is accompanied by increased phosphorylation of endogenous mouse Tau and of human Tau.P301L at the AT8 site. The timing remarkably coincides with the activation of the GSK3 kinases by intramolecular tyrosine phosphorylation and with the hippocampus-dependent cognitive deficits of APP.V717I and biAT mice. Cognition is already impaired at young adult age (age 4–6 months), well before the onset of amyloid deposition or Tau pathology, sensu stricto. Young adult biAT mice are severely impaired even in less demanding tests, that is, novel object recognition, passive inhibitory avoidance, and conditioned taste aversion [77].

Similar to humans suffering progressive senile types of dementia, ageing severely impacts the clinical phenotype of the bigenic mice, particularly of the biAT mice with progressive reduction in body weight, loss of ambulation, and reduced fur condition following the more early cognitive and behavioral impairments. The comparative evolution in both bigenic strains with ageing point to comparative underlying mechanisms, adding support to the hypothesis that amyloid triggers the tauopathy by increasing GSK3 activity [77, 118, 119].

8. Hypothesis: Tau, Phospho-Tau, Tau-P*, Oligomers, Fibrils, and Tangles

"...a stage, and all the...merely players"
W. Shakespeare 1598.

The molecular characterization of protein species intermediate between the monomers prepared by biochemists and the large fibrils observed postmortem by the pathologists is a hot topic in current neuroscience research and for many proteins that are proven or surmised to be implicated in neurodegenerative disorder [15, 120–123]. Probably, amyloid is the most advanced in this respect, although no single intermediate Aβ-oligomer is yet accepted as the only or even major toxic species. Relying on thermodynamics and mass-action laws, one can even predict that a single species will never be identified as the proven cause of all the distress in AD brain over a period of 20 years, or more.

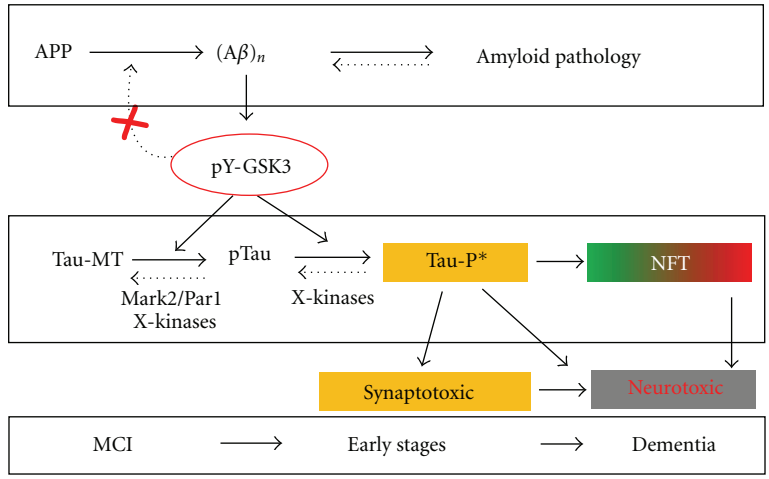

Figure 2: Schematic view of relations between amyloid and Tau in Alzheimer's disease. The interpretation hinges on the concept that the production of the Tau-P* intermediates are the central molecular species in the overall process. The incorporation of GSK3 as a major coupling link between the two pathologies is underscored essentially by *in vivo* observations in the mono- and bigenic mouse models discussed in the text. Protein Tau is a very soluble, naturally unfolded protein that in physiological conditions is located mainly in axons and attached to microtubules, denoted as Tau-MT. Activation by amyloid of both GSK3 kinases, together with other kinases including Mar2/Par1, gradually transforms Tau-MT into a pool of soluble, phosphorylated Tau (pTau) in the cytoplasm. Because of their delocalization, the pTau species undergo further posttranslational modifications, mainly including additional phosphorylation but eventually also nitrosylation, acetylation, truncation, all causing the transition into the conformational protein Tau species that we previously denoted as Tau-P* [11]. This as yet molecularly undefined intermediate likely represents soluble low-order aggregates that cause the early synaptic defects and cognitive problems typical for all tauopathies, with brain-region specificity as discussed in the text. The escape of Tau-P* from normal elimination via the proteasome and/or by autophagy leads to its accumulation, which by mass-action results in aggregation into NFT. We primarily consider NFT not to be detrimental for neurons, and initially they can even constitute a relative safety measure, as they reduce the free levels of Tau-P* and thereby its negative actions. Conversely, in the long term, the progressive accumulation of Tau-P* into more NFT deposition in neuropil and soma must invoke negative effects and eventually result in axonal and dendritic defects that culminate in neurodegeneration. This process is schematically reflected by the green-to-red color gradient of the background of the NFT box in the scheme.

Pathological and experimental evidence increasingly questions the direct link between intraneuronal accumulation of Tau-aggregates and the neuronal degeneration observed in the later phases of dementia.

Many neurons that accumulate NFT in aged human brain and in transgenic mouse brain are not marked by characteristic morphological signs of cellular death. Conversely, many neurons that do bear such markers do not appear to have a significant load of high-order Tau filaments [44, 124].

Therefore, both tangle formation and neuron loss should be considered as dissociated processes, at least in time, but likely also in actual underlying mechanisms (Figure 2). Direct assessment of the relation of tangles and neuronal function by electrophysiology of individual neurons ex vivo, concomitant with structural analysis of dendritic branching and spines, did not correlate with NFT bearing [125]. Similarly, the findings in mammalian models was also observed in invertebrate Tau models [126, 127]. The justified conclusion must be that other, less aggregated or even soluble forms of protein Tau are responsible for neuronal dysfunction and by extension for failing synaptic plasticity and cognition. More and more, NFTs are regarded to function as the intraneuronal sink for excess phosphorylated protein Tau that was released from the axonal microtubules and after delocalization and extra phosphorylation is unable to regain its normal physiological position and function. By forming large fibrillar aggregates, neurons are protected from toxic effects of soluble, smaller aggregates [128, 129], a mechanism that was even proposed as a potential therapeutic approach [130]. Obviously, the cytoplasmic sink is limited in capacity and this protective measure cannot but be limited in time, eventually resulting in the damage and death of the neuron, exemplified by the presence of ghost tangles in the later stages of tauopathy [16].

Tau species responsible for cognitive dysfunction were tentatively identified as multimers of apparent Mr 170 kDa, present in FTD and AD brain, and correlating with memory index and motor deficits in tauopathy models [91]. Furthermore, Tau oligomers prepared with Aβ oligomers as initial seed invoked memory impairment and synaptic and mitochondrial dysfunction in wild-type mice [131]. Invertebrate transgenic models, for example, drosophila that express wild-type or mutant Tau isoforms also concluded to soluble cytosolic Tau species as accountable for toxicity [48, 132].

Conversely, our own experiments with AAV-based vectors in wild-type mice revealed early damage mainly to dendrites by human wild-type and mutant Tau alike, accompanied by extensive phosphorylation and smaller Tau-aggregates of Tau, but without formation of larger fibrils [47, 133]. The situation led us to postulate that a phosphorylated intermediate, most likely a dimer or small aggregate that we termed Tau-P*, is responsible for cognitive dysfunction by

synaptic toxicity [19]. Our hypothesis hinges on the concept that the production of Tau-P* molecules constitutes for the neuron the tipping-point of whether or not to form the protective large aggregates. If for some reason, this is not possible, the accumulation of Tau-P* molecules will damage the neuron and impair synaptic function and plasticity, causing cognitive dysfunction (Figure 2).

This concept incorporates different signaling mechanism hinging on GSK3, and without abandoning the amyloid hypothesis. In the light of *in vivo* observations in transgenic mice, discussed in the foregoing sections, we maintain the GSK3 kinases center-stage as important link between the two pathologies in AD, reconciling the amyloid and Tau doctrines [77, 92, 119]. Activation of both GSK3 kinases by aberrant Aβ production and/or APP processing [134] is not the primary cause of the disease, but a major link to turn on the phosphorylation-cascade of protein Tau and the formation of Tau-P* intermediates. We realize that one kinase cannot suit all the known phosphorylation sites on protein Tau, while also the transformation of Tau-P* to NFT must depend on unknown factors (Figure 2). Moreover, the relation to the early and late cognitive impairment must depend on a host of as yet unknown molecular factors, termed X-factors before [19, 113] that must be held liable for the clinical symptoms in AD. Different factors in different individuals are expected to help explain the variable onset and evolution, as well as the variable cognitive and behavioral symptoms despite similar, although also variable brain pathology.

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Review Article

Lessons from Tau-Deficient Mice

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Both Alzheimer's disease (AD) and frontotemporal dementia (FTD) are characterized by the deposition of hyperphosphorylated forms of the microtubule-associated protein tau in neurons and/or glia. This unifying pathology led to the umbrella term "tauopathies" for these conditions, also emphasizing the central role of tau in AD and FTD. Generation of transgenic mouse models expressing human tau in the brain has contributed to the understanding of the pathomechanistic role of tau in disease. To reveal the physiological functions of tau *in vivo*, several knockout mouse strains with deletion of the tau-encoding *MAPT* gene have been established over the past decade, using different gene targeting constructs. Surprisingly, when initially introduced tau knockout mice presented with no overt phenotype or malformations. The number of publications using tau knockout mice has recently markedly increased, and both behavioural changes and motor deficits have been identified in aged mice of certain strains. Moreover, tau knockout mice have been instrumental in identifying novel functions of tau, both in cultured neurons and *in vivo*. Importantly, tau knockout mice have significantly contributed to the understanding of the pathophysiological interplay between Aβ and tau in AD. Here, we review the literature that involves tau knockout mice to summarize what we have learned so far from depleting tau *in vivo*.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia, characterized by a progressive decline of cognition due to synaptic and neuronal loss [1]. Despite intensive research into the cause of AD, there is no cure available to date, and current treatment options are limited to symptomatic relief [2]. This becomes even more alarming in the light of over 35 million AD patients worldwide, a number estimated to double by 2050 [3].

AD brains display two hallmark lesions upon autopsy: amyloid β (Aβ)-containing plaques and neurofibrillary tangles (NFTs). Aβ results from β- and γ-secretase-mediated proteolytic cleavage of the Aβ-precursor protein (APP) [4, 5]. NFTs are made up of hyperphosphorylated forms of the microtubule-associated protein tau [6]. In contrast to AD, FTD presents with tau pathology in the absence of an overt Aβ pathology. FTD is the second most prevalent form of dementia occurring before the age of 65 [7–9]. FTD describes a heterogeneous group of neurodegenerative disorders, including Pick's disease (PiD), frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), argyrophilic grain disease (AGD), corticobasal degeneration (CBD), and progressive supranuclear palsy (PSP). Sharing similar protein deposits, these disorders are characterized by a broad spectrum of clinical symptoms including behavioural changes, language abnormalities, and motor dysfunction (reviewed in [7, 9]). While in familial cases of AD (FAD), mutations were found in the APP- and the presenilin genes 1 and 2 [10], the latter being part of the γ-secretase complex [11], mutations in the MAPT gene were found in familial FTD [12, 13].

The tau protein has been discovered in 1975 as a protein with the ability to induce microtubule (MT) formation [14]. The tau-encoding gene *MAPT* is located on human chromosome 17q21 [15]. There are 6 tau isoforms, between 352 and 441 aminoacids in length, encoded by 11 exons in humans, with alternative splicing of exons 2, 3, and 10 [16]. They differ by either the presence or absence of up to two amino-terminal inserts (2N) and by containing either three (3R) or

four (4R) MT-binding repeats (MTB). While 3R isoforms are predominant during embryonic brain development, the normal adult brain has approximately equal levels of 3R and 4R isoforms [17]. Changes in this ratio have been linked to the pathogenesis of tauopathies, with increased 4R levels in AD and high amounts of 3R tau in PiD [18]. For comparison, mice and rats express only three different 4R isoforms of tau, but lack 3R tau [19].

Tau is expressed predominantly in neurons, where it is enriched in axons. Tau is either bound to microtubules, the inner side of the plasma membrane, or is unbound [20]. Besides stabilizing microtubules, tau has been implicated in the regulation of motor-driven axonal transport [21, 22]. Other possible functions of tau include cellular signalling, neuronal development, neuroprotection, and apoptosis [3, 23]. Furthermore, we have shown that tau is also present in dendrites at low levels, where it is involved in postsynaptic scaffolding [3, 24].

In AD and FTD, tau becomes increasingly phosphorylated at both physiological and pathological sites (referred to as "hyperphosphorylated") [25]. This aberrant phosphorylation detaches tau from microtubules, thereby probably compromising its microtubule-stabilising functions (loss of physiological function). At the same time, hyperphosphorylation of tau is the first step in the formation of toxic aggregates (gain of toxic function) and eventually of NFTs [26]. Hyperphosphorylated tau accumulates in the soma of neurons, giving rise to increased dendritic levels of tau [24, 27, 28]. However, there is good evidence that elevated levels of soluble tau already contribute to neuronal dysfunction prior to its deposition [29, 30], including for example, disruption of axonal transport [31–34] and impairment of mitochondrial function [35, 36].

The identification of pathogenic mutations in AD and FTD has led to the generation of multiple transgenic animal models that recapitulate important aspects of the human disease [37]. Transgenic mouse models, including those with human tau expression, have become the major *in vivo* tool in AD/FTD research (reviewed in [37]). In addition to tau overexpressing mouse strains, several tau knockout strains have been generated. Their contribution to the understanding of tau is reviewed in this paper.

2. Tau Knockout Mice

Expression of tau in cell lines resulted in elongated process formation, while tau reduction using antisense RNA suppressed axon elongation in cultured neurons [38]. Based on these findings in cells, tau depletion in mice was eagerly awaited with expectations of marked effects on neuronal systems *in vivo*. Surprisingly, four independently generated (conventional) tau knockout lines presented with no overt phenotype [39–42]. Only when aged did tau knockout mice develop behavioral impairments and motor deficits [43, 44].

In 1994, Harada and colleagues reported the first tau knockout mouse line (Figure 1(a)) [39]. The mice are viable and macroscopically normal. While immunohistochemical analysis did not show changes, electron microscopical analysis revealed decreased microtubule density in axons, together with reduced cross-bridging between parallel microtubules, and between microtubules and the plasma membrane. Interestingly, neurons from this particular strain showed normal axonal development in culture [39, 45]. The lack of tau was associated with an up to 2 fold increase in the microtubule-associated protein 1A (MAP1A) expression in 7-day-old, and a 1.3 fold increase in adult tau knockout mice, possibly compensating for the absence of tau [39]. Although MAP1B levels were reportedly normal in this tau knockout line, cross-breeding of tau with MAP1B knockout mice exacerbated hypoplastic axon tracts, disorganized neuronal layering, and impaired maturation of primary neurons of MAP1B mice [45].

In 2001, two additional tau knockout lines have been published. Tucker and colleagues generated tau knockout mice by integrating GFP-encoding cDNA into exon 1 of *MAPT*, resulting in expression of a GFP fusion protein with aminoacids 1 to 31 of tau, together with deletion of endogenous tau expression (Figure 1(b)) [41]. While the original report did use heterozygote tau knockout mice to image GFP expressing neurons, as well as sorting neurons for *in vitro* analysis, tau function has not been studied. Nevertheless, this particular strain has been used in several subsequent studies to identify novel functions of tau [24, 46].

Dawson and colleagues generated tau knockout mice by homologous recombination replacing exon 1 with a neomycin expression cassette (Figure 1(c)) [40]. Again, these mice are viable and display no overt anomalies. Similar to previous tau knockout mice [39], MAP1A levels were approximately 2-fold increased at birth, but declined with brain maturation. In fact, MAP1A levels were similar in wildtype and tau knockout mice at 12 months of age [40]. Hence, MAP1A may compensate for loss of tau during early brain development, but not in the mature brain [39, 40, 42]. Contrary to previous studies, primary neurons obtained from this tau knockout strain showed slowed maturation with reduced neurite length throughout all developmental stages and reduced axon length of stage 3 neurons [40].

The most recent tau knockout mouse has been established by Fujio and colleagues in 2007 [42]. They introduced a neomycin cassette in reverse orientation flanked by flippase recognition targets (FRTs) into exon 1 of *MAPT* (Figure 1(d)). Similar to previous tau knockout mice, they are viable and show no overt anomalies. MAP1A levels were increased as previously reported for other tau knockout strains [40].

Taken together, in three of four independent tau knockout strains, MAP1A is increased around birth but not in adult brain (NB: MAP1A levels have not been determined in the fourth strain), suggesting early but not late compensation for loss of tau by MAP1A [39, 40, 42]. On the other hand, neuronal maturation has been examined in two of four tau knockout lines, with different results [39, 40, 47]. Here, analysis of additional lines may provide clarification. Differences between the different tau knockout strains may be explained by different genetic backgrounds used.

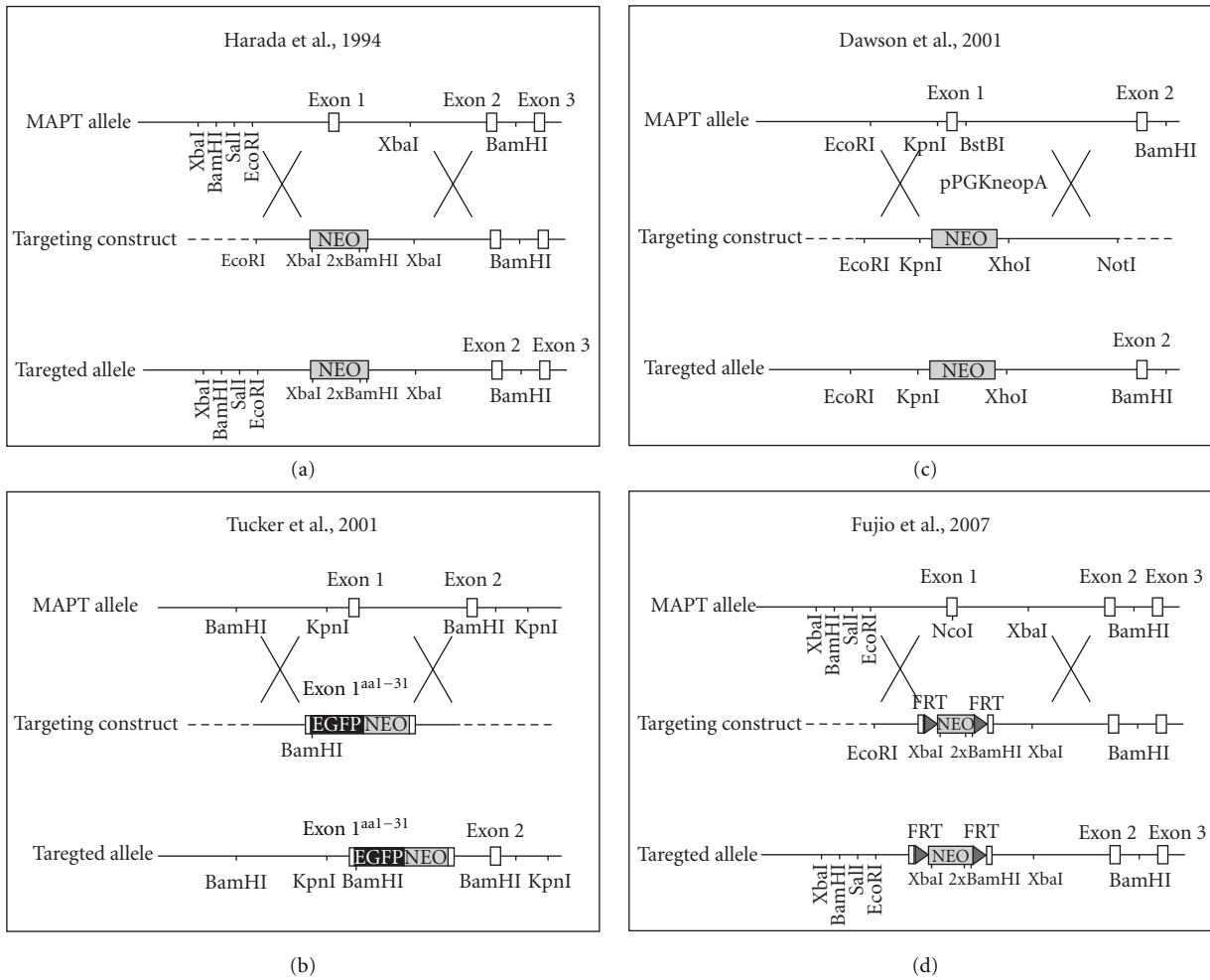

Figure 1: Homologous recombination strategies for the generation of different tau knockout mice reported by (a) Harada and collegues [39], (b) Tucker and colleagues [41], (c) Dawson and colleagues [40], and (d) Fujio and colleagues [42].

3. Deficits in Tau Knockout Mice

Neuronal dysfunction and neurodegeneration in tauopathies is due to the toxicity of pathologically modified tau and/or loss of physiological tau function [48]. While the reported tau knockout strains presented without overt phenotype when young [39–42], one-year-old mice of one tau knockout strain [39] showed muscle weakness in the wire-hanging test, reduced balancing in the rod-walking test, hyperactivity in new environments and impaired contextual fear conditioning [43]. Interestingly, muscle weakness was already detectable in heterozygote tau knockout mice. The spatial learning ability of this tau knockout strain, however, presented normal, when tested in the eight-arm radial maze and the Morris water maze [43]. Normal performance by 7- and 12-month-old tau knockout mice in the Morris water maze has since been independently confirmed in another strain [49, 50]. The latter strain also performed normal in the radial water arm maze and on the Rotarod at 12 months of age [50]. However, Lei and colleagues recently identified more complex motor deficits in the same tau knockout strain at 12 months of age, with increased turn time in the pole test, reduced performance on the Rotarod and decreased locomotion in the open field test [44]. These deficits were associated with reduced numbers of tyrosine hydroxylase-positive substantia nigra (SN) neurons [44]. Interestingly, mutant tau overexpression also results in a loss of SN neurons in a mouse model of FTD with Parkinsonism [33], suggesting that tau levels are critical for this neuronal population; however, the exact role of tau herein remains unknown. Taken together, tau knockout mice appear to develop motor deficits with increasing age, suggesting that loss of physiological tau function may contribute to the motor deficits observed in tauopathy patients. Further, tau knockout mice show no overt memory deficits in spatial memory tasks, which is consistent with sole tau pathology in humans, such as in FTD, where memory function is not or only mildly compromised.

Cantero and colleagues examined the local field potential from various cortical regions and hippocampus of tau knockout mice to determine if delayed axonal maturation of tau knockout neurons observed *in vitro* [40] could possibly

affect neuronal circuit formation *in vivo* [51]. They found slower theta rhythms of the hippocampus and reduced gamma synchronization between cortical brain regions in tau knockout mice, which may suggest impaired circuit formation. To this end, the morphological correlates of these findings remain unknown, but dysfunctional neuronal networks have been implicated in the pathogenesis of AD [52]. Furthermore, tau knockout mice have abnormal sleep-wake cycle, with increased wakefulness periods and reduced nonrapid eye movements [53]. Taken together, tau may play a role in neuronal circuit formation.

4. Tau Knockout Mice with Human Tau Expression

Overexpression of human tau derived from a human P1-derived artificial chromosome (PAC) in mice results in expression of all 6 human tau isoforms in mouse brains in the absence of any neuropathology [54]. Interestingly, crossing of PAC tau transgenic mice with tau knockout mice results in accumulation of hyperphosphorylated tau and formation of sarkosyl-insoluble 3R, but not 4R tau as early as 2 months of age [46]. Furthermore, 12-month-old PAC tau transgenic mice on a tau knockout background present with memory deficits in the Morris water maze task and perturbed LTP formation [55]. These aged mice are further characterized by neuronal loss together with ventricle enlargement and reduced cortical thickness [56]. Similarly, depletion of mouse tau exacerbated tau pathology in transgenic mice expressing double mutant human tau [57]. Together, this suggests that endogenous mouse tau prevents pathological alteration of transgenic human tau. The underlying mechanisms remain, however, elusive.

Sennvik and colleagues generated knockin mice by inserting cDNA that encodes the longest human tau isoform (2N4R) into exon 1 of the *MAPT* gene [58]. Surprisingly, neuronal numbers were increased in the hippocampus of these mice, as a result of increased neurogenesis and neuronal survival during development. This is accompanied by an improved performance in the novel object recognition task. Similar to other tau-deficient strains [40], maturation of primary neurons is delayed in primary cultures from these human tau knockin mice, probably because expression of human tau is only detectable after 10 days *in vitro* (DIV). Interestingly, at 4 DIV, human tau knockin neurons show proliferative markers, which disappear at the onset of human tau expression at 10 DIV and neuronal maturation [58]. Although mechanistically unclear, this may suggest anti-proliferative and prodifferentiation effects of human 2N4R tau.

5. Gene and Protein Regulation in Tau Knockout Mice

Tau depletion is associated with a 2 fold increase in MAP1A levels in newborn mice, but they are reduced to normal levels thereafter [39, 40]. This possible early compensation of the loss of tau by MAP1A has been reported together with the first tau knockout mice [39]. Since then, tau knockout mice have been subjected to both educated guess approaches and unbiased screening methods to identify additional genes and proteins that are deregulated, to further understand tau's role *in vivo*.

Tau has been shown to interact with histone deacetylase 6 (HDAC6), a tubulin deacetylase, via the microtubule binding domain of tau and the SE14 domain of HDAC6 that mediates its enzymatic activity [59]. Perez and colleagues showed that tau, in particular aggregated tau, inhibits HDAC6 activity *in vitro*, together with reduced levels of acetylated tubulin in primary tau knockout neurons, suggesting that tau regulates HDAC6 activity [60]. Another class of proteins that forms complexes with tau is 14-3-3, a group of scaffolding proteins [61–63]. However, the absence of changes in levels and interaction with microtubules of different 14-3-3 isoforms in tau knockout mice suggest that the interaction between tau and 14-3-3 is rather only relevant under conditions with increased levels of unbound tau [42], given that the interaction involves the microtubule-binding domain of tau that is normally preoccupied with tubulin [61].

Unbiased gene expression analysis revealed several genes that are changed in tau knockout compared to wildtype mouse brains [47, 64]. Oyama and colleagues identified several mRNAs that are deregulated in brains of tau-deficient mice [64]. Of those, Gem GTPase, a regulator of Rho signaling and inducer of cellular process formation, was significantly increased in tau knockout brains. In cells, tau suppressed the activity of Gem GTPase via its microtubule-binding domain, suggesting that tau may be involved in regulating Gem GTPase downstream signaling [64]. In another mRNA screening, de Barreda and colleagues found increased BAF57 mRNA and protein levels in the hippocampus of tau knockout mice [47]. BAF57 interacts with coREST, which in turn activates the transcriptional repressor REST and consequently the expression of neuronal specific genes [65]. Here, tau may act as a nuclear regulator of gene expression. Accordingly, tau can be isolated from nuclei of hippocampal cells [47, 66].

Taken together, analysis of deregulated genes and proteins in tau knockout mice proves valuable for the identification of novel tau functions. Advanced screening methods, such as next-generation sequencing, may provide further insights into tau-dependent processes in the future.

6. Protection from Aβ Pathology

According to the amyloid cascade theory, Aβ is upstream of tau pathology in the pathogenesis of AD [67]. This has been reproduced in mutant tau transgenic mouse models with NFT formation, by crossing them with Aβ-forming APP transgenic mice [68], or injecting Aβ into their brains [69], both resulting in increased NFT pathology. Interestingly, in 2002, Rapoport and colleagues provided the first evidence that tau is also needed for Aβ to cause its toxicity in neurons *in vitro*, as suggested by resistance of primary cultured neurons from tau knockout mice to Aβ exposure [70]. It was not until 2007, when Roberson and colleagues reproduced this finding *in vivo*, by crossing Aβ-forming APP

transgenic mice, which display premature mortality and memory deficits, on a tau knockout background [49]. Both hetero- and homo-zygote tau deficiency rescued premature mortality and prevented memory deficits in APP transgenic mice. Mechanistically, this protection appeared to be conferred by reduced susceptibility to excitotoxicity in tau knockout mice [49]. Excitotoxicity describes a signalling cascade that is induced by overactivation of NMDA receptors (NMDARs) that results in neuronal damage and death, and excitotoxicity has been implicated as a pathomechanism underlying neurodegeneration induced by Aβ in AD [71]. We reproduced the protection from Aβ-induced premature mortality and memory deficits, using independent APP transgenic, and tau knockout mice, also showing that reduced susceptibility to excitotoxicity of the latter underlies this protection [24]. Furthermore, we used expression of a dominant-negative truncation mutant of tau to prevent deficits in APP transgenic mice. Together with the tau knockout mice, we were able to show for the first time that tau is critically involved in postsynaptic NMDAR downstream signalling, by localizing the Src kinase Fyn to dendrites, where it mediates coupling of NMDAR complexes to postsynaptic scaffolding proteins and therefore signalling cascades. Reduced postsynaptic Fyn levels in tau-deficient or truncated tau expressing mice results in uncoupling of NMDARs from excitotoxic downstream signalling and therefore prevention of Aβ mediated toxicity [3, 24]. Hence, Aβ, Fyn and tau may orchestrate neuronal damage in AD mouse models [24, 72, 73], suggesting a critical role of tau in the pathogenesis of AD [3]. This data is consistent with previous reports on the preventive effects of Fyn depletion and accelerating effects of Fyn expression on the deficits in APP transgenic mice [74, 75]. Further, supporting the protective effects of tau depletion, Shipton and colleagues showed recently that Aβ-mediated impairment of LTP in hippocampal slices of wildtype mice is prevented in tau knockout mice [76]. LTP formation *per se* was normal in tau knockout mice [76], consistent with normal excitatory postsynaptic potential (EPSP) recordings in two different tau knockout strains [24, 73]. To this end, while several studies showed protection from Aβ toxicity by knocking out tau [24, 49, 70, 77], one other study showed increased pathology in aged mice [50], possibly reflecting different effects based on the usage of different APP transgenic lines.

The exact downstream mechanisms involved in mediating protection from Aβ toxicity in tau knockout mice remains to be shown. These may include axonal transport, which is known to be regulated by tau [22]. While basal axonal transport rates are unaffected in tau knockout neurons [77, 78], impairment of axonal transport of mitochondria and TrkA-containing vesicles induced by Aβ is prevented in tau-deficient neurons [77]. Furthermore, increased Aβ-formation is associated with increased activity of GSK3β, a known tau kinase [79, 80]. GSK3β overexpression in the brain of mice results in degeneration of the dentate gyrus, but this is significantly ameliorated when crossed with tau-deficient mice [81]. Tau knockout neurons also show increased resistance to heat shock [82]. While Hsp70 levels were increased upon heat shock in both wildtype and tau knockout neurons, Akt phosphorylation was delayed together with a virtual absence of GSK3β activation. Whether Akt/GSK3β signalling plays a role in preventing Aβ toxicity in tau knockout mice remains to be shown. If other tau kinases or phosphatases are involved also remains to be shown. Furthermore, protection of tau knockout neurons may be conferred by nuclear tau [83].

The protection from Aβ toxicity in tau knockout mice seems to be rather specific, since it does not prevent deficits in models of several other neurodegenerative disorders. Accordingly, crossing of mutant SOD1 expressing mice, a model of amyotrophic lateral sclerosis (ALS), on a tau knockout background, does not prevent weight loss and death [73]. Furthermore, motor deficits of mouse models of Parkinson's disease (PD) are not prevented on a tau-deficient background [84]. The PD models used were striatal injection of 6-hydroxydopamine and transgenic expression of α-synuclein, neither of which showed improvement. Similarly, tau depletion does not protect from deficits induced by intracranial administration of prions [84]. Tau depletion is associated with silver positive spheroids in yet another APP transgenic mouse strain when aged [50]. Finally, knocking out tau even exacerbated the phenotype of NPC deficient mice, a model of Niemann-Pick disease type C, suggesting a role of tau in regulation of autophagy [85].

Taken together, tau reduction prevents mice from specific Aβ-mediated deficits, supporting a central role of tau in mediating Aβ toxicity in the early pathogenesis of AD. However, tau depletion does not generally prevent from neurodegenerative conditions, suggesting distinct mechanisms.

7. Concluding Remarks

To this end, tau knockout mice have significantly contributed to unraveling novel functions of tau under physiological condition and its role in disease. While key findings have been reproduced in independent tau-deficient strains, others, such as delayed axonal maturation [40], remain to be confirmed in alternative strains. Differences may also be due to the usage of different genetic backgrounds with possible confounding effects. Understanding the differences might contribute to a broader knowledge about the physiologic function of tau, which may be translated to understanding the mechanisms of tauopathies.

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Review Article

MicroRNAs and the Regulation of Tau Metabolism

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Abnormal regulation of tau phosphorylation and/or alternative splicing is associated with the development of a large (>20) group of neurodegenerative disorders collectively known as tauopathies, the most common being Alzheimer's disease. Despite intensive research, little is known about the molecular mechanisms that participate in the transcriptional and posttranscriptional regulation of endogenous tau, especially in neurons. Recently, we showed that mice lacking *Dicer* in the forebrain displayed progressive neurodegeneration accompanied by disease-like changes in tau phosphorylation and splicing. Dicer is a key enzyme in the biogenesis of microRNAs (miRNAs), small noncoding RNAs that function as part of the RNA-induced silencing complex (RISC) to repress gene expression at the posttranscriptional level. We identified miR-16 and miR-132 as putative endogenous modulators of neuronal tau phosphorylation and tau exon 10 splicing, respectively. Interestingly, these miRNAs have been implicated in cell survival and function, whereas changes in miR-16/132 levels correlate with tau pathology in human neurodegenerative disorders. Thus, understanding how miRNA networks influence tau metabolism and possibly other biological systems might provide important clues into the molecular causes of tauopathies, particularly the more common but less understood sporadic forms.

1. Introduction

The discovery of small noncoding miRNAs uncovered an intriguing additional level to the fine-tuning of the transcriptome and proteome. Since their discovery almost 20 years ago [1, 2], research has progressed considerably towards gaining a better understanding of the impact of the complex network of gene regulation by miRNAs on health and disease. This is well documented in the cancer field, for instance, where miRNAs are increasingly acknowledged as potential diagnostic and therapeutic agents [3, 4]. Like protein-coding genes, miRNA genes are transcribed from the genome mainly from RNA polymerase II (reviewed in [5]). To date, more than 1000 miRNA genes have been identified in humans and 750 in mice, some of which are specifically expressed in the brain [6–10]. In the cytoplasm, the endonuclease Dicer cleaves miRNA precursors (approximately 70 nucleotides in length) to generate mature (approximately 21 nucleotides in length) double-stranded RNAs. These are loaded as single-stranded RNAs into the RNA-induced silencing complex (RISC), composed of Argonaute (Ago) proteins, to negatively regulate gene expression, albeit some exceptions have been documented [11, 12]. This regulation is achieved through binding with imperfect complementarity mainly to the 3′ untranslated region (3′UTR) of target messenger RNAs (mRNAs), leading to translation inhibition or degradation. Both *in vitro* and *in vivo* studies have shown that alterations of a single miRNA (or miRNA family) could have profound effects on hundreds of target genes [13, 14], thus possibly implicating multiple biological pathways.

Abnormal phosphorylation and insoluble deposition of tau are observed in more than 20 neurodegenerative disorders, collectively known as tauopathies (reviewed in [15]). In Alzheimer's disease (AD), the most common tauopathy, hyperphosphorylated tau accumulates in the somatodendritic compartment of neurons, aggregates, and finally forms

neurofibrillary tangles (NFTs). Other tauopathies include frontotemporal lobar degeneration (FTLD), Pick's disease, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and progressive aphasia, all of which are characterized by neuronal and/or glial tau inclusions.

Although the exact physiological role of tau remains under scrutiny, it is proposed to function to promote microtubule assembly, stabilization, spacing, and parallel-ordered organization, which are necessary for axonal transport and neurite outgrowth (reviewed in [16]). Tau binds to several proteins (reviewed in [17]) and therefore could participate in various other paradigms, including targeting the Src kinase Fyn to dendrites [18]. In the central nervous system, tau is expressed as six isoforms, resulting from inclusion or exclusion of alternative exons 2, 3, and 10 [19] (reviewed in [20]). Mutations in or surrounding tau exon 10, leading to an imbalance in tau isoforms with 3 or 4 microtubule-binding domains, can cause familial FTLD-17. Thus, tau missplicing can cause neurodegeneration and dementia in adulthood. Changes in tau isoforms have also been observed in various other tauopathies, including PSP and Pick's disease or myotonic dystrophy (reviewed in [21]); however, the underlying mechanisms involved in abnormal tau splicing and neurodegeneration remain ill-defined. Moreover, whether splicing abnormalities function upstream or concomitantly with tau hyperphosphorylation to promote neurodegeneration remains an open debate.

To date, several groups have identified factors involved in the regulation of tau splicing. These include regulatory sequences (cis-elements) within or around tau exon 10 as well as specific regulatory proteins (trans-acting factors) (reviewed in [20]). Similarly, a number of enzymes have been proposed to regulate tau phosphorylation (reviewed in [22]). Although these studies have been insightful, they were mostly based on artificial and/or overexpression paradigms, which makes it difficult to extrapolate these observations to endogenous tau.

In 2006, Bilen et al. [23] showed a remarkable enhancement of tau-mediated cell death in Drosophila cells upon suppression of miRNA maturation. Specifically, the authors showed that retinal degeneration caused by the expression of normal or mutant (R406W) human tau *in vivo* was enhanced by loss of R3D1/loquacious, a double-stranded RNA binding protein that is required for the activity of Dcr-1 (Dicer homologue) in miRNA processing. Interestingly, upregulation of *bantam* suppressed tau-induced degeneration, suggesting that this miRNA could mitigate tau-induced neurotoxicity. However, as *bantam* is not conserved in humans, it is tempting to speculate that other miRNAs play a similar role in the mammalian brain. More recently, a neuronal miRNA, miR-128, was shown to modulate the expression of BAG2, a cochaperone potentially involved in tau degradation and aggregation in cultured COS-7 cells and in primary neurons [24]. Our recently published data indicate that conditional knockout (cKO) of *Dicer* (resulting in a global reduction in miRNA production) in the adult mouse brain results in disease-like hyperphosphorylation of endogenous tau [25]. Moreover, the *Dicer* mutant mice display changes in tau exon 10 splicing [26], as seen in various tauopathies including PSP and Pick's disease. In this paper, we highlight salient observations with regard to these studies and highlight outstanding questions related to miRNA research in tauopathies.

2. Tau Phosphorylation Regulation by the miR-15 Family

Tau is a phosphoprotein that contains more than 80 potential phosphorylation sites (reviewed in [27]). It is generally well accepted that tau phosphorylation is important for microtubule binding, whereas phosphorylation causes tau to detach from microtubules. Hyperphosphorylation, defined as increased phosphorylation of physiological sites and additional phosphorylation at pathological sites, characterizes insoluble aggregates of tau. However, in its unphosphorylated form, under thermal stress, tau localizes to the nucleus, where it protects DNA from double-stranded DNA damage [28]. A phosphorylated pool of tau could also localize to somatodendritic compartment as well as the dendritic spine to modulate neuronal plasticity and glutamatergic transmission [18]. Tau phosphorylation is very sensitive to intrinsic and extrinsic changes (e.g., heat, cold, stress, and starvation) [29–32]. Thus, any changes in the delicate balance between the tau kinases and phosphatases could have serious biological consequences, and the identification of these regulatory enzymes *in vivo* is of particular importance. It is noteworthy that some of those central key kinases also regulate indirectly tau splicing through phosphorylation of splicing factors [33–36]. Together, deregulation of kinase and/or phosphatase activity could be dually detrimental towards tau splicing and phosphorylation, which synergistically would promote tau aggregation.

As general posttranslational regulation of gene expression, miRNAs are potential modulators of kinase, phosphatase, and/or splicing factor expression. While studying the effects of *Dicer* loss in the brain, we observed significant changes in endogenous tau phosphorylation and splicing [26]. This was demonstrated at the RNA and protein levels using, RT-PCR, 2D electrophoresis and tau phospho-specific antibodies. Because of using the Cre-LoxP system, Dicer inactivation was limited to neurons, and in particular postmitotic pyramidal neurons. It is noteworthy that only a few studies have documented changes in endogenous tau phosphorylation *in vivo*, as most biological models express exogenous and/or mutated human tau. Remarkably, several phosphoepitopes related to disease, including serine 422, were increased in the *Dicer* mutant mice when compared with controls [25]. Unfortunately, given the rather quick lethality associated with *Dicer* loss (approximately 4 weeks), we could not determine whether tau hyperphosphorylation concurred before or after neurodegeneration. Nevertheless, these results provide a proof of concept that miRNA haploinsufficiency causes abnormal tau hyperphosphorylation and missplicing.

As stated above, several tau kinases and phosphatases have been identified, some of which are believed to contribute significantly to tau hyperphosphorylation *in vivo*. In attempt to identify such enzymes in the *Dicer* cKO mice, we performed whole-genome microarrays and western

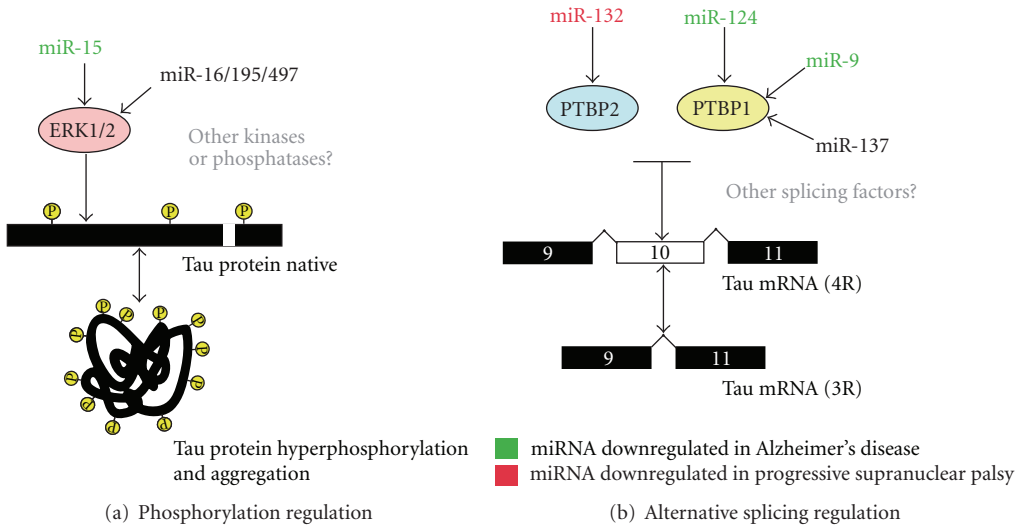

Figure 1: Potential role of miRNAs in tau metabolism regulation. (a, b) Two models are shown demonstrating how specific miRNAs could be involved in the regulation of tau phosphorylation (and aggregation) and/or tau exon 10 alternative splicing. Note that some miRNAs are affected in disease conditions (in green, downregulated in AD; in red, downregulated in PSP). Whether other miRNA target effectors are involved in the physiological and/or pathological regulation of tau metabolism remains to be explored. Any changes in the level or function of these miRNAs could have serious biological consequences, including tau hyperphosphorylation and aggregation and an imbalance in tau microtubule-binding repeats (encoded in tau exon 10, giving rise to either four 4R-tau or 3R-tau). Regulation of tau exon 10 splicing by PTBP1 or PTBP2 may be direct or may implicate other coregulators such as TDP-43 or PSF, both of which have been shown to either regulate PTBP2 expression or regulate PTBP splicing and repress tau exon 10 inclusion [26, 52–54].

blot analyses. These experiments led to the identification of ERK1/MAPK3 (and possibly ERK2/MAPK1) as a major regulator of neuronal tau phosphorylation *in vivo*, at least in this model. In line with this observation, several ERK1-dependent epitopes were hyperphosphorylated in the *Dicer* mutant mice. Of course, several other enzymes can potentially contribute to tau phosphorylation misregulation in this and other models, and in particular disease conditions in humans. In line with this hypothesis, a number of proposed tau kinases, including GSK3β and JNK/MAPK8, are prone to miRNA regulation in various cell types [37, 38]. Nevertheless, it is interesting to observe that ERK phosphorylation is increased in tau-positive neurons in AD and other tauopathies [39–41]. In addition, ERK is essential for brain development and involved in neuronal death [42, 43]. Specific gene knockout mouse models are required to assess the role of these proteins in the regulation of tau phosphorylation and neurodegeneration *in vivo*.

Although it is a conceptually crude experimental approach, the *Dicer* cKO mice provide a unique and unbiased model to study global miRNA function in the brain. To identify miRNAs involved in the regulation of ERK1 (and consequently tau phosphorylation), we used several prediction programs that are available online, including TargetScan (http://targetscan.org/). This program identified a number of potential miRNA-binding sites in the 3′UTR of ERK1. Our functional assays provided the validation that several members of the miR-16 family (miR-16, -15, -195, -497) could directly modulate endogenous ERK1 and tau phosphorylation in neuronal cells *in vitro*, including rat primary neurons. Of mention, both endogenous miR-16 and tau are enriched in distal axons of sympathetic neurons [44]. Intriguingly, miR-15a and miR-15b are downregulated in AD brain and cerebrospinal fluid, respectively [25, 45, 46], providing clinical relevance for these observations. Moreover, miR-15 targets the proapoptotic protein Bcl-2, whose protein levels are increased in AD [47–49]. In addition, miR-16 overexpression could regulate APP expression *in vivo* in the mouse brain [50]. Taken together, these observations highlight the potential importance of the miR-16 family in AD development by regulating cell survival, amyloid production, and tau phosphorylation (Figure 1). Interestingly, TargetScan predicts more than 1000 human target genes for miR-16, several of which are associated with networks related to cell death, cellular organization, and molecular transport (S. S. Hébert, unpublished observations). Notably, among the high-scoring predicted targets are miRNA-processing regulators such as TNRC6B [51]. It will be interesting to see whether loss of miR-16 family members *in vivo* recapitulates, at least in past, the observed effects on ERK and, most importantly, tau phosphorylation *in vivo*.

3. Tau Alternative Splicing Regulation by miR-132

As discussed above, abnormal regulation of tau exon 10 splicing can cause disease. It is interesting to note that *Dicer* deficiency in the adult brain is also associated with changes in tau splicing [26]. Using a similar strategy as above (e.g., bioinformatics, microarrays, literature search, western blot

analysis, etc.), we identified miR-132 and the neuronal splicing regulator PTBP2 as potential regulators of endogenous tau exon 10 splicing in neurons. These results are consistent with previous findings linking tau exon 10 splicing regulation by PTBP1 *in vitro* [55].

While not discussed in detail in our study, other miRNAs, including miR-124 and miR-9, could also regulate endogenous tau exon 10 splicing in neuronal cells by targeting specific regulatory and/or splicing factors [26]. Both miRNAs are downregulated in AD [56–58], which could have important consequences for tau metabolism, at least in certain biological contexts (Figure 1). For instance, downregulation of miR-9 is observed in the presence of exogenous Aβ in mouse primary neurons [59]. Whether this or other miRNAs function as intermediates between Aβ peptides, tau missplicing and hyperphosphorylation remain an exciting possibility. Interestingly, differential splicing of the tau transcript has also been reported in AD [60, 61].

PSP is a cause of late-onset atypical parkinsonism described by Steele et al. [62]. Dementia is also a common feature at the end stage of the disease. Neuropathologically, PSP is characterized by neuronal loss, gliosis, and NFT formation. Glial fibrillary tangles have also been described. In these patients, tau aggregates are mainly composed of tau with 4 microtubule-binding domains (4R-tau) [13, 15]. Using PSP as a model disease, we identified miR-132 to be selectively downregulated in pathological conditions. Interestingly, PTBP2 protein (but not mRNA) levels were increased in PSP patients and correlated significantly with miR-132 expression [26]. These experiments provide unprecedented molecular links among abnormal tau splicing, hyperphosphorylation, and sporadic tauopathies. Interestingly, changes in PTBP1 and PTBP2 levels, and by extension alternative splicing patterns, have been documented in human diseases, including neurodegenerative disorders [63, 64]. On the basis of these observations, it is tempting to speculate that miRNAs could contribute significantly to several aspects of tau metabolism and neuronal dysfunction in various diseases.

4. Outstanding Questions

Although the above-mentioned studies are interesting, many questions remain unanswered. For instance, what are the biological and clinical significance of these findings? Are other miRNAs involved in tau metabolism regulation? Can miRNAs be used as diagnostic and possibly therapeutic agents for sporadic tauopathies? Without a doubt, these and other questions will require extensive followup studies in various models, from cells to animals to humans.

Abbreviation

3′UTR: 3′ Untranslated region.

Conflict of Interests

The authors declared that there is no conflict of interests.

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Review Article

Drosophila Models of Tauopathies: What Have We Learned?

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Aggregates of the microtubule-associated protein Tau are neuropathological hallmark lesions in Alzheimer's disease (AD) and related primary tauopathies. In addition, Tau is genetically implicated in a number of human neurodegenerative disorders including frontotemporal dementia (FTD) and Parkinson's disease (PD). The exact mechanism by which Tau exerts its neurotoxicity is incompletely understood. Here, we give an overview of how studies using the genetic model organism *Drosophila* over the past decade have contributed to the molecular understanding of Tau neurotoxicity. We compare the different available readouts for Tau neurotoxicity in flies and review the molecular pathways in which Tau has been implicated. Finally, we emphasize that the integration of genome-wide approaches in human or mice with high-throughput genetic validation in *Drosophila* is a fruitful approach.

1. Introduction

For more than a century, the fruit fly *Drosophila* has been used to unravel major biological questions. The fruit fly has played crucial roles in deciphering various developmental signaling cascades such as the Notch, Wingless, and Hedgehog pathways. In addition, studies using *Drosophila* have contributed to a wide range of topics in neurobiology including neurodevelopment, behavior, circadian rhythms, learning and memory, synaptic transmission, and neurodegeneration [1, 2]. Since most basic molecular and cell biological mechanisms are conserved between humans and *Drosophila* and since ~70% of all human disease genes have an evolutionary conserved fly homolog, studies in flies have also provided valuable insights into the biology of human disease [3]. During the last decade, *Drosophila* has gained attention as a model system for common human neurodegenerative brain disorders [4]. In general, these models are based on the misexpression of human proteins such as α-synuclein [5], Tau [6, 7], and TDP-43 [8] that are present in the neuropathological hallmark lesions of patients with Parkinson's disease (PD), Alzheimer's disease (AD), frontotemporal dementia (FTD), and amyotrophic lateral sclerosis. Interestingly, expression of these proteins in flies results in neurotoxicity and the underlying molecular mechanisms appear to be largely protein- or disease-specific suggesting that this approach is useful.

Here, we review the contribution of *Drosophila* to the molecular understanding of Tau neurotoxicity, a central player in the AD-FTD spectrum of disorders [9]. We give a brief overview of the most commonly used genetic tools in *Drosophila* and summarize the different available Tau models and readouts for Tau neurotoxicity. Together, these studies paint a multifaceted picture of Tau being involved in a wide range of biological processes and highlight the complex role of Tau phosphorylation in mediating its toxicity.

2. Modeling Tauopathy in *Drosophila*

2.1. The Drosophila Genetic Toolkit in a Nutshell. Next to the fact that the fundamental molecular and cell biological aspects of neuronal biology are conserved between human

and *Drosophila*, the main advantage of the fly is its powerful, flexible, and extensive genetic toolkit. It essentially allows the expression, downregulation, or mutation of any gene, in a tissue- and time-specific manner [10].

A major tool is the binary GAL4-UAS system, which allows the expression of a genetic responder construct downstream of an Upstream Activator Sequence (UAS) driven by tissue-specific expression of the yeast GAL4 transcription factor [11]. The system can be used to either silence a gene using an RNAi-construct or to induce mis- and/or overexpression using a cDNA construct. Many driver lines have been generated, in which promoters of genes have been inserted upstream of the GAL4 sequence. The diversity of gene promoters makes it possible to target nearly all tissue or cell types. However, they usually do not allow time specificity. The most commonly used GAL4 drivers induce expression of the target protein from early developmental stages. Restricting expression to adulthood or to a defined time is relevant when modeling late-onset degeneration. For this reason, modifications of the GAL4-UAS system were developed in *Drosophila* and allow a tight regulation of transgene expression. For example, the TARGET system uses a temperature-sensitive mutant of the yeast GAL4 repressor GAL80 [12]. $GAL80^{ts}$ is active at low temperatures and suppresses GAL4 activity. To activate GAL4-induced gene expression in adulthood, adult flies are moved to 30°C, a temperature at which $GAL80^{ts}$ becomes inactivated and no longer inhibits GAL4 activity. In addition expression is reversible and shut off when shifting the flies back to lower temperature. Another system of conditional gene expression in *Drosophila* is called the Geneswitch system [13]. It consists of the pharmacological activation of a RU486-sensitive GAL4-derived transcription activator. The yeast GAL4 DNA binding domain has been fused with a mutated human progesterone receptor-ligand binding domain and with the transcriptional activation domain of the human p65, a member of the NFkB family [14]. The chimeric fusion protein is activated by RU486, binds to UAS sequences, and activates the transcription of downstream sequences. For RU486 induction, RU486 is added to *Drosophila* food [13].

Among the other genetic tools of *Drosophila* are transposons, which are mobile genetic elements, in which the transposase has been replaced by other sequences, such as UAS sequences to generate enhancer traps and GFP sequence that can be spliced to generate protein traps for example [10]. The main advantage is that these elements can be easily mobilized and insert randomly in the genome. If the transposon disrupts the gene sequence in which it is inserted, it can generate *loss-of-function* alleles of the gene. The imprecise excision of the transposon can also be used to generate genomic null mutations [10]. Different transposons such as P-element, PiggyBac, or Minos elements with complementary bias in their insertion site are now used to cover the whole *Drosophila* genome [15]. Null alleles can also be generated by chemical mutagenesis or X-ray radiation. Other powerful techniques are based on mitotic recombination, which can be used in a controlled manner to generate homozygous-mutant tissue in a heterozygous background. This allows determining the function of developmentally lethal genes in adult tissues [16]. All these tools give to researchers using *Drosophila* the possibility to perform in-depth reverse genetic studies as well as large-scale forward genetic screens, enabling the identification of novel biological pathways in an unbiased manner [17].

2.2. Tau Genetic Reagents. At least 37 constructs have been used to generate transgenic *Drosophila* Tau strains (Table 1). Tau cDNAs are most frequently inserted downstream of a UAS promoter although some Tau cDNAs are inserted downstream of the eye-specific *gl* promoter enabling simultaneous and independent expression of other UAS-constructs [7]. Tau transgenes were first used to improve neuronal labeling in morphological studies [18–20] until Williams and coworkers showed that these constructs induce neurodegeneration characterized by axonal loss and swellings [21]. Many *Drosophila* models were then generated using human Tau (hTau) (Table 1). Some are based on 0N3R, 0N4R, and 2N4R wild-type hTau isoforms [6, 7, 21], whereas others express mutated forms hTau, that cause autosomal dominant Tau-positive FTD, such as $hTau^{R406W}$, $hTau^{V337M}$, and $hTau^{P301L}$ [6, 22]. To explore specific mechanisms of hTau toxicity or dysfunction, transgenes with targeted mutations and truncations were also generated, including constructs which abolish or mimic hTau phosphorylation or proteolytic cleavage [23–31]. Together, these models explore the great diversity of tauopathies.

While most *Drosophila* studies on Tau neurotoxicity are based on overexpression of hTau, it is important to mention that *Drosophila* has a single *tau* gene/protein (dTau) [36]. Compared to the 6 human isoforms, which harbor either 3 or 4 C-terminal microtubule-binding domains (MTBD) and 0 to 2 N-terminal insertions, the dTau protein contains 5 MTBD with 46% identity and 66% similarity to the corresponding hTau region but no N-terminal insertions [36]. Homozygous dTau null *Drosophila* mutants are viable and fertile and display no obvious morphological or behavioral defects [33], although a microtubule-based defect in polarity has been shown in dTau null oocytes [37]. The absence of major defects in dTau null mutants may be due to redundancy with other microtubule-associated proteins such as Futsch, the MAP1B *Drosophila* ortholog. In agreement, the degenerative phenotype of hypomorphic *futsch* alleles was partially suppressed by dTau overexpression in the central nervous system [38]. Some constructs also express dTau, which have been used to compare the function and toxicity of endogenous dTau with hTau [32–34, 39]. These studies revealed similar degrees of neuronal dysfunction for dTau and hTau (see below) although genetic and physical interaction partners showed important differences between the two Tau proteins [39, 40].

2.3. Readouts of Tau Neurotoxicity and Dysfunction in Drosophila. The choice of a readout for Tau neurotoxicity or dysfunction results from a trade-off between the ease and speed of scoring a phenotype and its biological or pathogenic relevance. The *Drosophila* exoskeleton provides a wealth of external features, such as bristles and compound eyes, which

Table 1: Tau constructs available in *Drosophila*.

Constructs	Type	Origin
Drosophila constructs		
UAS-dTau	Wild-type	Mershin et al. 2004 [32]
UASp-dTau^{A}:mGFP6	Fusion	Doerflinger et al. 2003 [33]
UAS-dTau:1D4	Tagged form	Feuillette et al. 2010 [34]
Bovine constructs		
UAS-bTau	Wild-type	Ito et al. 1997 [18]
bTau$^{1-382}$:GFP	Fusion	Micklem et al. 1997 [35]
UAS-bTau:GFP	Fusion	Murray et al. 1998 [19]
UAS-bTau:lacZ	Fusion	Callahan and Thomas 1994 [20]
Human 0N3R construct		
UAS-hTau		Williams et al. 2000 [21]
Human 2N4R constructs		
gl-hTau	Wild-type	Jackson et al. 2002 [7]
gl-hTau^{P301L}	FTDP17 mutation	Karsten et al. 2006 [22]
UAS-hTau		Chatterjee et al. 2009 [23]
gl-hTau^{S2A} (S262A/S356A)	Phospho-deficient	Chatterjee et al. 2009 [23]
UAS-hTau^{S2A} (S262A/S356A)	Phospho-deficient	Chatterjee et al. 2009 [23]
gl-hTau^{S11A} (S46A/T50A/S199A/S202A/ S205A/T212A/ S214A/T231A/ S235A/S396A/S404A)	Phospho-deficient	Chatterjee et al. 2009 [23]
UAS-hTau^{S11A} (S46A/T50A/S199A/S202A/ S205A/T212A/S214A/T231A/ S235A/S396A/S404A)	Phospho-deficient	Chatterjee et al. 2009 [23]
UAS-hTau:FLAG	Tagged form	Kosmidis et al. 2010 [24]
UAS-hTau^{STA}:FLAG (S238A/T245A)	Tagged form Phospho-deficient	Kosmidis et al. 2010 [24]
Human 0N4R constructs		
UAS-hTau	Wild-type	Wittmann et al. 2001 [6]
UAS-hTau^{V337M}	FTDP17 mutation	Wittmann et al. 2001 [6]
UAS-hTau^{R406W}	FTDP17 mutation	Wittmann et al. 2001 [6]
UAS-hTau$^{R406W\ S2A}$ (S262A/S356A)	FTDP17 mutation Phospho-deficient	Nishimura et al. 2004 [25]
UAS-hTau$^{R406W\ S202A}$	FTDP17 mutation Phospho-deficient	Nishimura et al. 2004 [25]
UAS-hTau$^{T111A/T153A}$	Phospho-deficient	Steinhilb et al. 2007 MBC [26]
UAS-hTau$^{T175A/T181A}$	Phospho-deficient	Steinhilb et al. 2007 MBC [26]
UAS-hTau$^{T199A/T217A}$	Phospho-deficient	Steinhilb et al. 2007 MBC [26]
UAS-hTau$^{S202A/S205A}$	Phospho-deficient	Steinhilb et al. 2007 MBC [26]
UAS-hTau^{T212A}	Phospho-deficient	Steinhilb et al. 2007 MBC [26]
UAS-hTau^{S214A}	Phospho-deficient	Steinhilb et al. 2007 MBC [26]
UAS-hTau$^{T231A/S235A}$	Phospho-deficient	Steinhilb et al. 2007 MBC [26]
UAS-hTau^{S262A}	Phospho-deficient	Iijima-Ando et al. 2010 [28]
UAS-hTau$^{S396A/S404A}$	Phospho-deficient	Steinhilb et al. 2007 MBC [26]
UAS-hTau^{S422A}	Phospho-deficient	Steinhilb et al. 2007 MBC [26]
UAS-hTau^{AP5} (S202A/S205A/T212A/ T231A/S235A)	Phospho-deficient	Steinhilb et al. 2007 MBC [26]

Table 1: Continued.

Constructs	Type	Origin
UAS-hTau^{AP} (T111A/T153A/T175A/T181A/ S199A/S202A/S205A/T212A/ S214A/T217A/T231A/S235A/ S396A/S404A/S422A)	Phospho-deficient	Steinhilb et al. 2007 JNR [26]
UAS-hTau^{E14} (T111E/T153E/T175E/T181E/ S199E/S202E/S205E/ T212E/ T217E/T231E/S235E/S396E/ S404E/S422E)	Phosphomimetic	Khurana et al. 2006 [29]
UAS-hTau$^{K44Q/R230Q}$	Calpain-resistant	Reinecke et al. 2011 [30]
UAS-hTau$^{44–230}$	Calpain 17kDA proteolytic fragment	Reinecke et al. 2011 [30]
UAST-hTau$^{1–421}$	C-terminally truncated Tau	Khurana et al. 2010 [31]

Table 2: Readouts of Tau toxicity in *Drosophila*.

Organ/Tissue/Cells	Phenotypes	Promoters/Drivers
Eye	Roughness	GMR
	Photoreceptor cell viability	Gl
		Sev
Notum	Loss of bristles	Eq
Nervous system	Lethality	Elav
	Shortened lifespan	Appl
	Brain vacuolisation	Repo
	TUNEL positive cells	Gl
	Activated caspase3 positive cells	C472
	Loss of olfactory Learning and memory	C772
Motor neurons	Axonal transport defects	D42
	Locomotor deficits	OK6
	"juvenile" phenotype: loss of wing	Elav
	expansion and cuticle tanning	Burs12
		Appl

can be affected by genetic manipulations, and for which the resulting phenotypes can be scored simply and quickly in young flies by looking through a stereomicroscope. These readouts of neurotoxicity or dysfunction, especially the eye external morphology, have been successfully used in screens for modifiers of Tau pathology and other neurodegenerative diseases [41–43]. Here we give an overview of the different available readouts of Tau neurotoxicity and dysfunction in *Drosophila* (Table 2).

2.3.1. The Eye. Roughening of the eye is the most commonly used external phenotype to evaluate toxicity of neurodegenerative proteins, including Tau, in *Drosophila*. The fly eye consists of around 800 highly regularly implanted ommatidia, each containing 8 photoreceptor neurons. The eye is an excellent tissue to study the effect of organismal lethal genes as it is dispensable for viability. Due to its repetitive crystal-like pattern, it is ideal to identify mild external morphological defects upon expression of human neurotoxic proteins. It thus constitutes a genetically sensitized system that allows the identification of genetic modifiers by assessing roughening of the eye as a quantitative readout of neurotoxicity. The eye surface of the *Drosophila* eye is generated during the final stages of development and thus, this phenotype has a developmental component. However, it is associated with vacuolization in the underlying optic brain structures [7], a typical sign of degeneration in the fly nervous system. Interestingly, a pure degenerative phenotype can be established in the *Drosophila* eye by assessing the viability of photoreceptor neurons over time in living adult flies. This is possible thanks to the cornea neutralization technique, which consists of visualizing photoreceptor neurons directly through the cornea of a living anesthetized fly, immersed in a medium with a refractive index comparable to the fly cornea, such as water [44]. Photoreceptor neurons are detected based on Rhodopsin autofluorescence or on the expression of fluorescent protein such as GFP. Because it is an *in vivo* method, the same fly can be analyzed at several time points during adult lifespan and exhibit progressive degeneration. The usefulness of related fluorescence-based techniques in neuronal degeneration was further illustrated by Gambis, and colleagues [45]. In a clonal screen using a derived method, called Tomato/GFP-FLP/FRT method, several mutants were identified that induced progressive photoreceptor loss. These methods have not yet been tested in the context of Tau neurotoxicity or other proteinopathies. Another potentially useful method that has not yet been used in *Drosophila* models of Tauopathies is the electrophysiological analysis using electroretinogram recordings (ERG). ERG analysis consists of recording the electrophysiological activity of the retina upon exposure to light. This activity is sensitive to PR degeneration. ERG measurements can be used to show a progressive loss of neuronal functioning [46].

2.3.2. The Notal Bristles. Neurotoxicity assays based on external fly features, such as eye roughness, are important as they are easily scorable and thus suited for high-throughput genetic screening. Interestingly, a novel bristle loss phenotypic assay for Tau neurotoxicity was described recently [47]. The *Drosophila* notum (part of the thorax) harbors around 200 bristles, which are sensory organs, connected at the base with the dendrite of a sensory neuron. Overexpression of different variants of hTau in the notum using the notum-specific Eq-GAL4 driver leads to bristle loss. hTau toxicity can be quantified by simply counting the bristles. This was done for wildtype hTau, phosphomutant hTau, and phosphomimetic hTau in addition to FTD-associated mutant hTau. In general, the sensitivity of the eye and the bristles to different variants was comparable. The notal bristle assay thus constitutes an interesting complementary and quantitative model to molecularly dissect Tau neurotoxicity.

2.3.3. Lifespan and Lethality. The ultimate consequence of (neuro)toxicity is the death of the whole organism, which can also be scored relatively easily by counting the number of surviving flies over time. Depending on the rearing conditions, wild-type flies live around 60 to 80 days and lifespan can be easily used as a quantifiable readout of Tau toxicity [6, 31, 47–50]. On the other hand, lethality can occur during development before eclosion of adult flies. Using the pan-neuronal Appl-GAL4 and Elav-GAL4 drivers, Tau-expressing flies exhibited pupal lethality, which can also be quantified and serve as a readout of Tau toxicity [49, 51, 52].

2.3.4. Brain Degeneration. Neuronal degeneration in *Drosophila* can be demonstrated by the presence of vacuoles in brain tissue using histological and immunohistochemical methods. Interestingly, vacuoles are found in the brains of hTau-expressing flies [6, 7, 32] and, although labor-intensive, the number of vacuoles can be used as a quantitative readout of hTau neurotoxicity [29, 53, 54]. Neuronal cell death can be further detected using specific stainings. The TUNEL technique is frequently used to detect apoptosis in brains of hTau-expressing flies [25, 29, 50, 54]. Alternatively, immunostaining of activated cleaved caspase-3 can be performed to demonstrate apoptotic cell death [50, 53].

2.3.5. Axonal Transport Assays. The main function of Tau is to bind to microtubules. Hence, it has been hypothesized that Tau toxicity or dysfunction could result from a defect in axonal transport. *Drosophila* is well suited to study axonal transport because fluorescent (GFP) fusion proteins tagging transport vesicles can be expressed in larval motor neurons, which are accessible for imaging in living intact animals [55]. In addition, larval locomotor phenotypes have been described for mutants that affect axonal transport, such as kinesin or dynein mutants [56, 57]. Immunostainings against Synaptotagmin were first performed to assess axonal transport in Appl-GAL4 larvae expressing hTau [51, 52]. It enables the visualization of synaptic vesicles along axons. Mudher and colleagues then used a GFP/neuropeptideY fusion protein to image vesicle axonal transport through the body wall of living larva expressing hTau in motor neurons (D42-GAL4 driver) [40, 48, 55]. Talmat-Amar and colleagues further enriched the analysis with a Synaptotagmin-GFP construct expressed in larval motor neurons (OK6-GAL4 driver) and with kymographs [49]. Kymographs consist of visualizing the movements of all vesicles within a nerve segment over time. They allow the measurement of the kinetic parameters of vesicular movement such as instant velocity or pausing time.

Axonal transport disruption can also be detected at the level of the whole organism. Two readouts, locomotor deficits and the "juvenile" phenotype, have been used depending on the neurons in which hTau is expressed. First, Mudher and colleagues analyzed successfully the motor function of larvae expressing hTau in motor neurons (D42-GAL4) using contraction, crawling, line-crossing, and righting assays [55]. These assays enabled them to compare the effects of 3R hTau and dTau on locomotor functions or the interaction between hTau and Aβ42 [40, 48]. Locomotor functions can also be assessed in adult flies using a climbing or negative geotaxis assay. Flies display a strong negative geotactic response. When tapped to the bottom of a vial they rapidly climb to the top of the vial, and most flies remain there. Locomotor dysfunction impairs climbing ability. Using this readout, locomotor dysfunction was quantified in Tau-expressing flies (D42-GAL4 and elav-GAL4) [53, 55]. Second, a strong defect in axonal transport can also result in altered release of neuropeptides or neurohormones at neurohemal release sites. When bursicon neurons are affected, loss of the bursicon neurohormone prevents wing expansion and cuticle tanning just after fly eclosion [58]. This immature unexpanded wing phenotype is easily visible and quantifiable in young flies. Several drivers, OK6-GAL4, Burs12-GAL4, Appl-GAL4 or elav-GAL4, are expressed in bursicon neurons [51, 59, 60], and it has been shown that the inhibition of axonal transport *per se* in bursicon neurons affects wing expansion [49]. This phenotype has been used as a readout to compare the toxicity of phosphorylation mutants of hTau and to show a genetic interaction between hTau and *Appl,* the fly APP homolog [49, 51].

2.3.6. Learning and Memory Assays and Mushroom Body Ablation Phenotypes. Tauopathies affect the cognitive functions of patients. In *Drosophila*, olfactory memory can be used as a readout for assessing impaired cognitive functions in hTau-expressing flies. Olfactory learning and memory relies on neurons located in a distinct region of the fly brain called mushroom bodies [61]. Tau expression can be targeted to these neurons using the pan-neuronal driver elav-GAL4 or the late pupal, adult mushroom body-specific drivers C492-GAL4, C772-GAL4 [24, 32]. By testing response to attractive and repulsive odors, olfactory learning and memory has been standardized and can be measured in transgenic flies. An aversive phototaxis suppression assay has also been used to measure learning and memory function in Tau-expressing flies [32, 53]. Strikingly, pan-neuronal overexpression of hTau leads to selective and nearly complete ablation of the mushroom bodies [24, 32]. These phenotypes can

also be used to identify genetic interactors of Tau dysfunction/toxicity.

3. Pathogenic Mechanisms of Tauopathies

3.1. Tau and Phosphorylation. Studies in *Drosophila* have revealed a highly complex role of Tau phosphorylation in mediating neuronal function or toxicity. The rough eye phenotype was the starting point for the Jackson lab, to investigate the role of Wingless signaling and *Shaggy* (*Sgg*, the fly GSK3β homolog) on hTau toxicity [7]. Overexpression of *Sgg* significantly enhanced hTau toxicity, even to the point that neurofibrillary tangle-like structures could be detected, while a loss-of-function allele of *Sgg* had a beneficial effect on toxicity. In order to investigate if hTau toxicity is mediated by Wingless signaling, genetic interaction between Tau and the downstream Sgg target *armadillo* (*arm*), which is inhibited by Sgg, was investigated. Unexpectedly, loss of *arm* rescued whereas misexpression enhanced the hTau rough eye phenotype independently of hTau phosphorylation. These results suggested that the *Sgg* interaction with hTau does not go through the canonical Wnt pathway and that Sgg directly or indirectly leads to phosphorylation of hTau (see also Section 3.5.1). A follow-up study further suggested an indirect effect since the mutant 2N4R hTau^{S11A} that cannot be phosphorylated by Sgg is still toxic [23].

The role of phosphorylation in Tau pathology was investigated by several labs [23, 25–27, 49, 62]. The Lu lab showed that, overexpression of fly PAR-1 kinase (MARK) in the eye induces a moderate eye phenotype, which was partially suppressed in a heterozygous deletion background of dTau. A strong synergistic enhancing effect was observed in a background expressing hTau^{R406W} [25]. Reduction of PAR-1 function or mutation of PAR-1 phosphorylation sites (S2A) was shown to reduce 0N4R hTau^{R406W} toxicity. Phosphorylation was also suggested to occur in a structurally ordered pattern. First S262 and S356 are phosphorylated which facilitates targets of Sgg to be phosphorylated. The Jackson lab independently showed that wild-type 2N4R hTau, in which serines S262 and S356 are substituted to alanines (S2A), displayed lower toxicity in the eye due to the inability of PAR-1 to phosphorylate the S2A tau at the two mutated serines [48]. However, the Lu and Jackson labs disagree about the priming effect of PAR-1 before Sgg phosphorylation. The Lu lab showed that the 0N4R hTau$^{R406W/S2A}$ construct is not phosphorylated at some Sgg sites and is refractory to toxic enhancement by Sgg overexpression [25], while the Jackson lab found that 2N4R hTau^{S2A}, although less toxic, was still phosphorylated at Sgg sites when Sgg is overexpressed [23]. The different conclusions by the two labs are unclear but may be due to the different isoforms used (0N4R hTau$^{R406W/S2A}$ versus 2N4RhTau^{S2A}). Altogether, these results demonstrate, in contrast to Sgg, a direct role for PAR-1 in mediating Tau toxicity.

In order to identify phosphorylation sites involved in toxicity, the Feany lab generated a number of 0N4R hTau constructs each having one or two Ser-Pro and/or Thr-Pro mutated to phosphoresistant alanines (Table 1) [27]. Although no significant reduction in toxicity was observed for any of these phospho-deficient constructs, a 0N4R hTau construct in which 14 Ser-Pro and Thr-Pro target sites are mutated to alanine (hTau^{AP}) displayed reduced neurotoxicity [26]. Accordingly, a phosphomimetic construct with the same 14 epitopes mutated to glutamate (hTau^{E14}) increased Tau toxicity, suggesting that toxicity relies on cooperation of different phosphorylation sites. However, using a 2N4R construct in which 11 Ser-Pro and Thr-Pro target sites are mutated to alanine (hTau^{S11A}) of which 9 overlapped with hTau^{AP}, the Jackson lab did not observe decreased toxicity in the eye [23]. The reasons for these discrepancies are not clear but might be related to the different hTau isoforms used, the number of mutated sites, or the differences in the mutated sites. The toxicity of the phosphomimetic Tau^{E14} construct has been further reported in mushroom body neuroblasts, whereas the phospho-deficient Tau^{AP}, 0N4R hTau$^{R406W/S2A}$, and 2N4R hTau^{S2A} were not toxic [24]. In mushroom body neuroblasts, Ser238 and Thr245 were also shown to be essential for 2N4R hTau toxicity [24].

Tau phosphorylation also affects neuronal function in the absence of neuronal loss/toxicity. The tau protein is known best, functionally, as a microtubule stabilizing protein. The fraction of Tau that is bound to microtubules is inversely correlated with its phosphorylation state. In the context of microtubule stabilization, hyperphosphorylation leads to *loss-of-function* effects while hypophosphorylation can lead to increased microtubule stabilization. How expression of hyperphosphorylated hTau affects microtubular integrity in the fly was elegantly investigated by the Mudher group [55, 63]. They found that, despite the presence of endogeneous dTau, overexpression of 0N3R hTau led to axonal microtubule breakdown. When expressed in flies, 0N3R hTau becomes hyperphosphorylated. Upon treatment with Li^+, a GSK3β inhibitor, phosphorylation was reduced and microtubule binding of hTau was increased. Interestingly, the same was shown for dTau, suggesting that hyperphosphorylated hTau can sequester endogeneous dTau. Furthermore, it was shown that the physical interaction between dTau and hTau occurred in a phosphorylation-dependent manner. In addition, the Mudher lab found that overexpression of hTau in motorneurons leads to defects in axonal transport without neurodegeneration. They investigated the role of hTau phosphorylation by Sgg on axonal transport. Pharmacologic inhibition of Sgg, with two different inhibitors, could reverse Tau-induced defects in axonal transport. Overexpression of hTau resistant to Sgg phosphorylation (Tau^{S11A}) was later shown to display a higher binding affinity for microtubules [23]. This suggests that pharmacologic inhibition of Sgg probably restores axonal transport by increasing the microtubule affinity of hTau. Later, Talmat-Amar and coworkers showed that at the level of axonal transport, hTau^{AP} was clearly more toxic than hTau^{WT} or hTau^{E14} and related to its capacity to bind more strongly to microtubules [49]. Taken together, these data suggest that microtubule affinity of hTau is most likely, at least in part, regulated by Sgg-dependent phosphorylation. Furthermore, it can be concluded that both hypo- and hyperstabilization of axonal microtubules has detrimental effects.

3.2. Tau and the Cytoskeleton

3.2.1. Binding of Tau to Microtubules. By nature, Tau is a microtubule-binding protein and therefore has a strong involvement in the regulation of the cytoskeleton. In *Drosophila,* endogenous dTau and exogenous bTau and hTau colocalize with microtubules *in vivo* [33, 35, 36]. dTau and hTau interact with microtubules in microtubule cosedimentation assays *in vitro* [23, 33]. dTau binds microtubules more strongly than 0N4R hTau or $hTau^{R406W}$ and $hTau^{V337M}$ [34]. This weak binding of hTau to microtubules depends on its phosphorylation status. The microtubule-bound hTau in the pellet was found to be hypophosphorylated, whereas hTau in the supernatant was phosphorylated [34]. In addition, using phosphomimetic and phospho-deficient hTau forms to assess the role of phosphorylation, it was shown that the majority of phospho-deficient $hTau^{AP}$ and $hTau^{S11A}$ proteins were pelleted with microtubules, whereas the pseudophosphorylated Tau^{E14} proteins remained mostly in the supernatant fraction [23, 34, 49]. Thus hTau expressed in *Drosophila* is phosphorylated, which prevents a strong binding to microtubules.

3.2.2. Functional Consequences of Deregulated Tau-Microtubule Interaction. One functional consequence of Tau deregulation is the alteration of the microtubule network. Overexpression of hTau has been associated with microtubule breakdown in peripheral nerves of L3 larvae [63]. In this model, the cytosolic phosphorylated hTau bound endogenous dTau and dissociated dTau from microtubules. This would be responsible for the disruption of the microtubule cytoskeleton as claimed by the Tau-microtubule hypothesis [64]. In loss-of-function experiments, both follicle and germline cells of dTau null ovaries did not display strong alterations in their microtubule network [33]. However, the polarity of dTau null oocytes was altered at stage 10, a phenotype similar to that of excessive PAR-1 overexpression [37]. In addition, dTau overexpression partially rescued the phenotype of PAR-1 overexpression, which suggests that dTau is involved in maintaining microtubule stability in the oocyte and that PAR-1 regulates oocyte polarity at least partly through dTau [37].

Another functional consequence of Tau deregulation is the alteration of axonal transport. Overexpression of bTau, 0N3R hTau, and dTau in third instar larvae results in large accumulations of synaptotagmin or GFP:neuropeptideY-tagged vesicles in motor neuron axons [40, 51, 55]. Coexpression of GSK3β and pharmacological inhibition of GSK3β, respectively, increased and decreased this phenotype, suggesting that Tau phosphorylation could enhance axonal transport disruption [55]. Although this study did not detect a vesicle motion defect, a later study also using 0N4R hTau showed an increase in the pausing rate of the vesicles within axons [49]. The pausing defect was drastically stronger using 0N4R $hTau^{AP}$, a phospho-deficient form that strongly binds to microtubules and affected mainly anterograde transport. Expression of $hTau^{AP}$ induced a juvenile wing inflation phenotype, similarly as downregulation of dynein and kinesin [49]. Expression of the pseudophosphorylated $hTau^{E14}$ did not disrupt vesicle motion and even slightly increased instant velocity [49]. Interestingly, a reciprocal regulation of Tau phosphorylation by axonal transport has been described [52]. A nonlethal reduction in kinesin-1-dependent axonal transport was associated with increased activated cJun N-terminal Kinase (JNK), hTau phosphorylation, hTau accumulation, axonal vesicle accumulation, and hTau toxicity [52]. This suggested that axonal transport defects can activate axonal stress kinase pathways leading to hTau phosphorylation, stabilization, and an increase in hTau-mediated neurodegeneration.

Taken together, these studies seem to converge on the notion that direct PAR-1-mediated Tau phosphorylation is directly involved in neurotoxicity, while the effects of GSK3β appear to be more indirect and related to neuronal functioning rather than toxicity.

3.2.3. Tau and the Actin Cytoskeleton. *cheerio* (fly ortholog of filamin), *chd64* (fly ortholog of transgelin-3), *jaguar* (fly ortholog of myosinVI), *paxillin*, 4 regulators of the actin network, were identified as modifiers of the Tau^{V337M}-mediated rough eye phenotype in a misexpression screen [43]. *cheerio* had been identified also in a previous similar screen [41]. Filamin-A and MyosinVI were further found to colocalize with fibrillary hTau protein in AD and FTD brains [65]. The Feany lab showed that actin might be a critical mediator of Tau-induced neurotoxicity [66]. They showed that $hTau^{R406W}$ interacts directly with F-actin in the fly brain. $hTau^{R406W}$ overexpression induced the accumulation of F-actin and the formation of actin-rich rods, which were similar to Hirano bodies found in AD. F-actin accumulation and the formation of actin-rich rods correlated with the degree of Tau-induced neuronal degeneration. Decreasing F-actin levels reduced neurotoxicity in the retina of hTau transgenic flies. This indicated that F-actin mediated hTau neurotoxicity [66]. In addition, whereas $hTau^{E14}$-induced retinal toxicity was clearly modified by genetically modulating the actin cytoskeleton, the $hTau^{AP}$-induced rough eye was not enhanced by coexpressing actin. This showed that actin changes occur downstream of hTau phosphorylation [66].

3.2.4. Tau and the Larval Neuromuscular Junction (NMJ). It has been shown that overexpression of dTau, 0N3R hTau, 0N4R hTau, and $hTau^{V337M}$ in larval motor neurons causes morphological disruption of NMJs characterized by satellite boutons [40, 43, 67]. In 0N3R hTau-expressing motor neurons, this is associated with abnormal endo/exocytosis characterized by decreased evoked synaptic potentials following high frequency stimulation [67]. The authors suggested that this may be due to a reduction in axonal transport of mitochondria resulting in a reduction of functional mitochondria in the presynaptic terminal [67]. No axonal transport defects were observed in $hTau^{V337M}$-expressing larval motor neurons but in this model, abnormally shaped NMJs were associated with loss of acetylated alpha-tubulin [43]. The authors suggested that disruption of the cytoskeleton network in presynaptic nerve terminals could constitute early events in

the pathological process leading to synaptic dysfunction in $hTau^{V337M}$ pathology.

3.3. Degradation of Tau. Dysfunction of protein degradation may favor accumulation of toxic Tau species. Several studies in *Drosophila* have analyzed Tau degradation. Endogenous dTau was first described as being not degraded by the proteasome pathway [68] although high-molecular-weight forms of hyperphosphorylated hTau were shown to be degraded by the proteasome [43]. *Hsp70/Hsp90-organizing protein homolog* (*Hop*), a scaffold protein for chaperones, has been identified as a suppressor of 2N4R hTau toxicity and has been proposed to facilitate clearance of hTau via the Ubiquitin-Proteasome System (UPS) [42]. Recently, nicotinamide mononucleotide (NAD) adenylyl transferase (NMNAT), a protein that has both NAD synthase and chaperone function, was shown to interact with phosphorylated hTau oligomers *in vivo* and promote the ubiquitination and clearance of toxic hTau species [53]. In contrast, other chaperones, *DnaJ-1*, *Csp*, and *Hsc70Cb*, have been identified unexpectedly as enhancers of $hTau^{V337M}$ toxicity [43]. Despite the cytosolic subcellular localization of Tau, expression of $hTau^{WT}$, $hTau^{R406W}$, and $hTau^{E14}$ in *Drosophila* brain triggers the unfolded protein response (UPR), a cell response that handles excess misfolded proteins in the secretory pathway causing endoplasmic reticulum (ER) stress [69]. Mild ER stress is protective in retinal degeneration [70]. The UPR similarly protected against tau neurotoxicity [69].

Alternative degradation processes to the ubiquitin-proteasome system include proteases and the autophagy-lysosomal pathway. Calpain A and B have been shown to cleave Tau and generate a toxic 17 kDa Tau fragment [30]. Mutations that disrupt endogenous calpain A or calpain B activity or expression of a calpain-resistant form of Tau $hTau^{K44Q/R230Q}$ in transgenic flies abrogated Tau toxicity *in vivo* [30]. The puromycin-sensitive aminopeptidase (PSA) has also been described as a modifier of Tau toxicity but as a suppressor [22]. Although the original study described that PSA digests directly Tau *in vitro* [22, 71], this idea has been challenged more recently [72]. As shown in a *Drosophila* polyQ disease model, the protective effects of PSA may instead be mediated through activation of autophagy [73]. Several components related to the autophagy-lysosomal pathway, *Atg6*, *Vha14*, *Vha44*, *white*, *brown*, *rosy*, *dynein light chain 2*, *benchwarmer/spinster,* and *cathepsinD,* have been identified as modifiers of Tau toxicity in *Drosophila* [31, 42, 43, 46, 62]. Loss-of-function mutations of *benchwarmer/spinster*, *white, brown,* and *cathepsinD* are associated with enlarged lysosomes and enhanced hTau toxicity [31, 46, 62]. Loss of *cathepsinD* is also associated with caspase activation and generation of a C-terminally truncated form of hTau, which is more toxic and less soluble [31]. This suggests that caspase cleavage of Tau may be a molecular mechanism through which lysosomal dysfunction and neurodegeneration are causally linked in Tauopathies.

3.4. Cell Death Pathways Associated with Tau Toxicity. Many studies have detected markers of apoptosis, such as TUNEL-positive staining, activated cleaved caspase 3, cleaved PARP, and abnormal accumulation of lamin in hTau-expressing tissue [7, 25, 29, 50, 53, 54, 66, 69]. Components of the apoptotic pathway, such as dIAP1 and FEM-1, were identified as modifiers of Tau^{V337M} toxicity [41]. In addition, overexpression of dIAP1, dIAP2, and the baculovirus caspase inhibitor p35 partially rescued the hTau-mediated rough eye phenotype [7]. These results strongly support a role for apoptosis in Tau-induced neurodegeneration in *Drosophila*. The pathways that activate apoptosis upon Tau toxicity have not been described yet. The study of *cathepsinD* mutant showed that lysosomal dysfunction could trigger caspase activation in fly Tau models [31]. Reactivation of the cell cycle downstream of Tau phosphorylation has also been shown to precede apoptosis in postmitotic neurons [29]. Fly brains overexpressing $hTau^{R406W}$ stained positive for the cell cycle markers PCNA and PH3. Inhibition and activation of the cell cycle, respectively, reduced and increased Tau toxicity but did not affect Tau phosphorylation. This indicated that cell cycle activation is a mediator of Tau toxicity acting downstream of Tau phosphorylation. This was confirmed by the use of the phosphomimetic $hTau^{E14}$, which induced increased cell cycle marker staining and toxicity in comparison with $hTau^{WT}$. The link between Tau phosphorylation and cell cycle activation was shown to be mediated by the TOR (Target of Rapamycin kinase) pathway [29]. Furthermore, oxidative stress has been shown to enhance Tau-induced cell cycle activation and toxicity [54]. Genetic or pharmacological inhibition and induction of oxidative stress reduced and increased $hTau^{R406W}$ toxicity. Oxidative stress did not act by altering Tau phosphorylation but enhanced cell-cycle activation [54].

3.5. Cell Signaling Pathways Modulating Tau Toxicity. Several cell signaling pathways have been shown to modulate Tau toxicity but the exact contribution of each pathway and their interactions remain to be clarified and confirmed.

3.5.1. The Wingless(Wg)/Wnt Pathway. Sgg/GSK3β, one of the major Tau kinases, belongs to the Wingless(Wg)/Wnt pathway. GSK3β increased hTau phosphorylation, induced hTau aggregation, and increased hTau toxicity [7]. In the Wg/Wnt pathway, Sgg/GSK3β inhibits armadillo/β-catenin, the downstream effector with TCF. Whereas one could expect that inhibition of armadillo and dTCF also contributed to GSK3β-enhanced hTau toxicity, loss of *armadillo* and *dTCF* rescued hTau rough eye phenotype whereas misexpression enhanced it. This showed that (1) GSK3β does not exert its primary effect via the canonical Wg pathway but, rather, via direct hTau hyperphosphorylation. (2) The Wg pathway contributes to hTau toxicity, possibly through a function in apoptotic cell death in degeneration associated with hTau [7]. However, mimicking the *Wg* signaling pathway by coexpression of dishevelled, an upstream component of the pathway, with $hTau^{WT}$ reduces Tau phosphorylation and Tau toxicity [48]. Therefore, the Wg pathway seems to be involved in Tau pathology but the role of each component of the pathway remains to be clarified.

3.5.2. The Mitogen-Activated Protein Kinase (MAPK) Pathways. Several members of the MAPK family have been involved in modulating hTau toxicity. From the cell stress response pathway, the stress kinase Hemipterous(Hep)/JNK was identified as an enhancer of the $hTau^{V337M}$-induced rough eye phenotype [41]. Overexpression of Hep/JNK also exacerbated 0N4R $hTau^{WT}$ toxicity without affecting the phospho-deficient $hTau^{AP}$, which suggested that Hep/JNK-induced increased hTau toxicity is mediated through hTau phosphorylation [27]. This mechanism has been involved in the stress-inducing nonlethal reduction of axonal transport [52]. Hep/JNK activation has also been detected upon oxidative stress and the extent of Hep/JNK activation correlated with the degree of hTau-induced neurodegeneration [54]. In this model, oxidative stress acted not to promote hTau phosphorylation but to enhance hTau-induced cell cycle activation [54]. Recently, a genetic screen for modifiers of 2N4R hTau toxicity identified components of the Extracellular Regulated Kinase (ERK)/MAPK and p38/MAPK pathways [42]. Loss-of-function alleles of *ksr* and *Neuroglian* (*Nrg*), both of which promote ERK signaling, suppressed hTau toxicity, suggesting that reducing ERK activity is beneficial. Overexpression of *Mekk1*, a MAP3K that leads to the phosphorylation and activation of p38/MAPK, was found to strongly enhance hTau toxicity. Cross-talk between these signaling pathways was shown and may also include interactions with the GSK3β signaling pathway [42]. Interestingly, in this study, some results suggested that hTau could have a signaling function and be able to regulate its own kinases. In addition, overexpression of 2N4R $hTau^{S11A}$ was previously shown to increase the activated form of GSK-3β (Sgg Y^{214}) in comparison with 2N4R $hTau^{WT}$ [23]. As suggested by Ambegaokar and coworkers, if Tau has the ability to regulate its own kinases, and if this regulation is phosphorylation-dependent, this would broaden our understanding of the role of Tau phosphorylation, which to date has been primarily associated with reduced microtubule-binding affinity [42].

3.5.3. The TOR Pathway. It has been shown that the TOR pathway links phosphorylated hTau to a toxic cell cycle activation in postmitotic neurons [29]. Overexpression of $hTau^{R406W}$ upregulated S6K phosphorylation, the target of TOR kinase. Pharmacological or genetic inhibition of *TOR* reduced $hTau^{R406W}$- and $hTau^{V337M}$-associated toxicity. The TOR-induced increase in Tau toxicity was suppressed by inhibiting cell cycle activation.

3.5.4. The DNA Repair Pathway. Recently, kinases from the DNA repair pathway have been involved in a toxic phosphorylation of Tau [28]. It has been shown that the *Drosophila* DNA damage-activated Checkpoint kinase2 (Chk2) phosphorylates hTau at Ser262 and enhances hTau toxicity [28]. In addition, the DNA repair response was increased by Aβ42 [74]. It is thus possible that the DNA repair pathway induced by Aβ triggers Tau phosphorylation and toxicity in the pathogenesis of AD.

3.5.5. The Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) Pathway in Glia. In glia, the JAK/STAT signaling pathway has been shown to be protective against Tauopathy [50]. Expression of 0N4R hTau in glial cells using the *repo* driver resulted in aged-dependent hTau phosphorylation, hTau aggregation, formation of hTau tangles and glial, and neuronal cell death. These phenotypes were associated with a progressive loss of JAK/STAT signaling. In addition, inhibiting or activating the JAK/STAT signaling pathway in glia enhanced and suppressed cell death in fly brain expressing hTau in glia [50]. The protective effect of the JAK/STAT pathway would occur downstream of Tau phosphorylation as modulating the JAK/STAT pathway did not change hTau phosphorylation [50].

3.6. Tau and APP/Aβ. Human Aβ42 and *Appl*, the *Drosophila* ortholog of APP, have been shown to synergistically increase Tau toxicity in *Drosophila*. Coexpression of bTau and *Appl* under the *ApplG1a* driver synergistically induced axonal transport defects in larval motor neurons, lethality at the pharate adult stage, and a juvenile phenotype at the adult stage [51]. Aβ42 expression strongly increased the rough eye phenotype of hTau-expressing flies, as well as vacuolization and the number of TUNEL-positive cells in the brain [66]. Aβ42 coexpression also exacerbated Tau-mediated disruption of axonal transport and synaptic structures, leading to locomotor defects and reduced lifespan [48]. This interaction shows that *Drosophila* is a relevant model for AD. Furthermore, studies in *Drosophila* gained insight into the synergistic link between Aβ42 and Tau. Double-transgenic flies coexpressing Aβ42 and either $hTau^{AP}$ or $hTau^{E14}$, the phospho-deficient and phosphomimetic hTau constructs, showed no clear changes in retinal toxicity, suggesting that the interaction between Aβ42 and hTau requires intact Ser/Thr phosphorylation sites on Tau [66]. Coexpression of hTau and Aβ42 increased hTau phosphorylation and treatment of flies coexpressing hTau and Aβ42 with LiCl suppressing the exacerbating effect of Aβ42 [48]. This suggests that GSK3β may be involved in the mechanism by which Aβ42 and hTau interact, potentially through hTau phosphorylation, to cause neuronal dysfunction [48]. Another mechanism has been proposed and involved the Checkpoint kinase2 (Chk2) from the DNA repair pathway [28, 74]. Coexpression of Aβ42 and hTau resulted in increased hTau phosphorylation at several sites including Ser262 and increased hTau toxicity. Mutating Ser262 to Ala prevented hTau phosphorylation at this site and alleviated Aβ42-induced Tau toxicity [74]. Ser262 is a target site of the DNA damage-activated kinase Chk2 [28]. A number of genes involved in the DNA repair pathway like Chk2 are increased in Aβ42 fly brains, and the induction of a DNA repair response is protective against Aβ42 toxicity [74]. These results suggest that activation of DNA repair pathways is protective against Aβ42 toxicity but may trigger hTau phosphorylation and toxicity in AD pathology [74].

3.7. Cell- and Isoform-Specificity of Tau Toxicity and Dysfunction. Discrepancies between studies suggest that mechanisms of Tau toxicity may be different depending on cell-types and Tau isoforms. A striking example comes from the phosphomimetic and phospho-deficient Tau forms (see Section 3.1). The phosphomimetic $hTau^{E14}$ is strongly toxic in the developing eye and in the mushroom body neuroblasts, in which the phospho-deficient $hTau^{AP}$ is not [24, 27, 29, 66], The opposite is true for vesicular motion in motor neuron axons, neurohormone release, and animal survival [49]. This is probably due to the state of differentiation of the Tau-expressing neurons and the critical role of axonal transport in these neurons. It was also shown that the phosphorylation status and stability of Tau depend on the subneuronal population in which Tau is expressed [75].

Besides cell-type specificity, hTau isoforms, FTD-related hTau mutant forms, and Tau orthologs seem to have toxic specificities despite common properties. The Skoulakis lab has compared the toxicity of different Tau isoforms in the embryonic neuroblasts that generate the mushroom body neurons, and the functional consequence on learning ability in the adult flies [24]. They observed that expression of 0N4R and 2N4R hTau neuroblasts strongly affected the development of mushroom body neuroblasts leading to the loss of mushroom bodies in the adult. Expression of the FTD-related 0N4R $hTau^{R406W}$ and $hTau^{V337M}$ were mildly toxic, whereas dTau, bTau, and 0N3R hTau were not toxic. By comparing bTau and hTau sequences, they identified two phosphorylation sites, Ser238 and Thr245, which were specific to hTau. Mutation to alanine of these sites fully suppressed hTau toxicity in mushroom bodies neuroblasts [24]. Hence they were able to determine the difference between bTau and hTau that is responsible for their specific effects in mushroom bodies neuroblasts. Other examples of isoform specificity have been reported outside the mushroom bodies. In motor neurons, $hTau^{V337M}$ did not alter axonal transport in comparison with $hTau^{WT}$ but rather affected NMJ morphology [43]. Overexpression of either dTau or hTau in the retina resulted in a similar rough eye phenotype. However, coexpression of PAR-1 with dTau led to lethality, whereas coexpression of PAR-1 with 0N4R hTau had little effect on the rough eye phenotype [39] and Par-1 coexpression increased 2N4R hTau toxicity in the eye [23]. The origin of the differences between these isoforms still need to be explored. It has to be noted that possible genetic interactions between ectopic hTau and endogenous dTau should also be considered. Endogenous dTau expression has been shown to modulate bristle loss in the fly notum induced by $hTau^{WT}$ [47]. The level of endogenous dTau expression may participate in the cell-type specificity.

The work on the different isoforms led the Skoulakis lab to highlight the distinction between Tau toxicity and Tau dysfunction (for reviews on this subject [76, 77]). Whereas expression of 0N4R hTau and 2N4R hTau was toxic and induced loss of mushroom bodies, expression of bTau and dTau did not affect the structure of the mushroom bodies but affected cognitive function [24]. Other phenotypes reported by other group are also more related to toxicity or dysfunction. Eye roughness, lethality, and decreased lifespan represent toxicity, whereas some axonal transport, behavioural, or synaptic defects represent dysfunction and were reported without toxicity [55]. The next question will be to understand what regulates Tau toxicity and Tau dysfunction. *Drosophila* has already given some cues. Phosphorylation of Tau may be a good candidate. Phosphorylation of Ser238 and Thr245 has been shown to be responsible for hTau toxicity in mushroom bodies neuroblasts [24]. Phosphorylation of Tau by PAR-1 has been shown to be toxic in the eye [25], whereas phosphorylation of Tau by GSK-3β seemed to reduce microtubule binding rather than be toxic and thus be related to dysfunction [23].

4. Genetic Screens in *Drosophila*: Perspectives

4.1. Forward Genetic Screens. To date, three forward genetic screens for hTau modifiers have been published, all using the hTau-associated rough eye as a readout for toxicity [41–43]. The first two screens reported were highly similar in design. Both screens were performed using the FTD-associated $hTau^{V337M}$ mutant form of Tau and both screens screened transposon insertions inducing misexpression [41, 43]. The transposon insertions, P{EP} and P{Mae-UAS-.6.11}, can induce both gain- and loss-of-function phenotypes, depending on the orientation of the insertion and the presence of a GAL4 driver. Although the screens were highly similar in design, the outcomes were considerably different likely because of differences in the screened transposon collections. The screen by Shulman and Feany identified kinases and phosphatases as most represented interactors [41]. Among these, genes previously shown to affect hTau phosphorylation were found to modify the rough eye phenotype. They identified *par-1*, the *Drosophila* homolog of MARK as a suppressor of hTau toxicity. Furthermore, two regulatory subunits of the known tau phosphatases, PP1 and PP2A, were identified. In the category of the kinases and phosphatases, *string* and *twine* were also found. Both are phosphatases, and when overexpressed, suppress Tau toxicity. This suggests that *string* and *twine* might dephosphorylate tau and hence reduce toxicity. Additionally, *thread* and CG9025 were identified. Both are inhibitors of apoptosis, and the effect of the transposon insertions is as expected. If apoptosis inhibition is enhanced, the hTau rough eye phenotype is suppressed. Other interactors hinted a role for the cytoskeleton; overexpression of *orbit*, *dfxr1*, and *cheerio* aggravated the tau-induced rough eye. The last category overlaps with the findings of Blard and colleagues in their very similar screen setup [43]. They identified *cheerio, Chd64, jaguar,* and *paxillin* as enhancers. Remarkably, all four of these genes are linked to the actin cytoskeleton. *Cheerio*, also identified in the screen of Shulman and Feany, encodes the *Drosophila* homolog of filamin. Filamins play an important role in stabilizing and cross-linking filamentous actin. A loss-of-function insertion in *Chd64*, the homolog of mammalian *transgelin-3,* was found to enhance the tau phenotype. In mammals, *transgelin-3* was shown to colocalize with filamentous actin and α-tubulin, but also with

Tau itself and MAP2. *Jaguar* was found to be homologous to mammalian class VI Myosins, which are motor proteins who transport their cargo towards the actin minus ends [43]. Paxillin is a regulator of the Rho family GTPases, Rac and Rho, which regulate actin cytoskeletal dynamics. Other interactors identified in the screen included following transcriptional regulators, CG33097, *dumpy,* and *nab,* but also several transporters (1.28, *vha44*, *ATPα*) were identified.

The most exhaustive screen was recently published by Ambegaokar and Jackson [42]. They screened two different P-element insertion collections. In addition to the EY collection, which can induce both gain- and loss-of-function effects, this group for the first time screened a loss-of-function collection consisting of 920 genomically mapped lethal P-elements. In total, 1905 lines were screened and 37 modifiers of tau toxicity were identified. To exclude suppressors that act on general apoptosis, each of the suppressors was tested for inhibition of apoptosis by crossing them to flies eye specifically overexpressing an inducer of apoptosis, *hid*. The modifiers were then tested for effects on the Tau phosphorylation state. No consistent effects were observed among enhancers or suppressors. To quantify the effects on eye morphology, volume calculations were performed on eyes of both enhancers and suppressors. The authors could show that for all suppressors eye volume was increased, the opposite was shown for the enhancers. Using the software tool "Endeavour-highfly," the investigators constructed a network that extrapolates the findings and suggests new pathways, cellular processes, and genes that might also be involved in pathology. A network with the predicted associations with statistical predictions $P < 0.001$ shows an interesting role for RNA processing, lysosomal degradation pathways, and, as expected, kinases and phosphatases. Five of the *in silico* predicted genes were tested for their ability to modify tau pathology *in vivo*. Both Tom34, a mitochondrial protein, and Csul, a protein involved in RNA trafficking, enhanced Tau pathology when gene function is reduced. Overexpression of both genes could induce a substantial rescue. A gene involved in RNA catabolism, *armi,* enhanced Tau pathology in both loss-of-function and gain-of-function approaches. *Upf1*and *Tom20*, involved in, respectively, RNA catabolism and mitochondrial function, did not exert an effect on external eye morphology of flies overexpressing hTau. This shows that the applied software prediction tool is able to successfully identify genetic interactors of hTau, based on results of a forward genetic screen.

4.2. Integration of Mammalian Genome-Wide High-Throughput Experiments with Functional Validation in Drosophila. Genome-wide high-throughput experiments such as genetic genome-wide association studies (GWAS) and transcriptomics have emerged during the last years as powerful strategies to identify novel biological disease pathways. However, given the vast amount of novel data generated in these experiments and the fact that the function of many of the identified genes or loci are not known, the biological interpretation of these studies is difficult. Recently, two very interesting strategies tackling this problem were published. The first study combines transcriptome analysis of mice overexpressing Tau with functional validation in *Drosophila* [22]. The second study starts with target identification in an AD GWAS study, followed by validation in the fly [78]. In both studies, *Drosophila* plays a central role in the functional validation of targets found in mice or patients.

Karsten and colleagues analyzed transcriptomes of different brain regions of mice overexpressing the most common FTD-associated mutant $hTau^{P301L}$. These mice phenocopy major pathological hallmarks of tauopathy including neurodegeneration in the spinal cord and cortex. Neurodegeneration becomes apparent when the mice reach 7 to 9 months of age but transcriptome analysis was performed at 6 months of age when no major pathological phenotypes are present, but low amounts of phosphorylated Tau can already be detected. In total, 31 probe sets were found to be differentially regulated in Tau mice compared to wild-type controls. An interesting hit was the puromycin-sensitive aminopeptidase (PSA) for which no neuroprotective role had previously been suggested. To further investigate the role of PSA and other genes, mutant alleles of the *Drosophila* homologs were tested in a fly tau model. Loss of-function of PSA enhanced while overexpression suppressed Tau toxicity. To further validate PSA as a causal gene in neuroprotection, PSA abundance was evaluated in both FTD patients and controls. Interestingly, in both patients and controls, PSA expression was fivefold higher in the cerebellum than in the cortex and a significant increase in PSA was found in FTD patients compared to controls [37].

Recently, the first study combining an AD GWAS study with functional validation in *Drosophila* was published [33]. The study started with a GWAS on an autopsy cohort of 227 individuals who were nondemented at recruitment. All individuals were yearly evaluated clinically. The GWAS identified 22 gene loci suggestive of being associated with AD ($P < 10^{-3}$). Of these 22 candidate genes identified, 19 genes had a clear homolog in *Drosophila*. In the second step, these genes were tested in a fly model that uses the Tau-induced rough eye as a read-out. Using a transgenic RNAi approach, each of these 19 genes were knocked down in flies overexpressing $hTau^{V337M}$. If fly strains were available, overexpression of the identified candidate genes was also tested. Six genes, when knocked down, displayed a clear genetic interaction with Tau, *B-spec*, *fne*, *Glut1*, *hs6st*, *dlg,* and *slit*. For three of them, the opposite interaction was observed with overexpression lines. When combined with statistically more powerful GWAS datasets, functional validation of GWAS hits using Tau and Aβ toxicity readouts in *Drosophila* will likely be more powerful and informative.

5. Conclusion

In this paper, we have attempted to give an overview of the many technical possibilities the *Drosophila* system offers to study the molecular, cellular, and physiological mechanisms by which Tau causes neuronal degeneration and dysfunction. The available studies reveal that Tau is involved in a wide range of cellular and biochemical processes. Regarding the role of phosphorylation of Tau in neuronal degeneration,

studies in *Drosophila* suggest that this link is highly complex and possibly less important than generally accepted. The most innovating results from *Drosophila* have and will continue to come from (1) unbiased high-throughput forward genetic screens that identify modifiers of Tau neurotoxicity and (2) studies that address the normal function the dTau gene. Finally, linking unbiased *Drosophila* to human genetics will likely identify important molecular mechanisms involved in Tau-mediated neuronal degeneration.

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Research Article

Tau and Caspase 3 as Targets for Neuroprotection

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The peptide drug candidate NAP (davunetide) has demonstrated protective effects in various *in vivo* and *in vitro* models of neurodegeneration. NAP was shown to reduce tau hyperphosphorylation as well as to prevent caspase-3 activation and cytochrome-3 release from mitochondria, both characteristic of apoptotic cell death. Recent studies suggest that caspases may play a role in tau pathology. The purpose of this study was to evaluate the effect of NAP on tau hyperphosphorylation and caspase activity in the same biological system. Our experimental setup used primary neuronal cultures subjected to oxygen-glucose deprivation (OGD), with and without NAP or caspase inhibitor. Cell viability was assessed by measuring mitochondrial activity (MTS assay), and immunoblots were used for analyzing protein level. It was shown that apoptosis was responsible for all cell death occurring following ischemia, and NAP treatment showed a concentration-dependent protection from cell death. Ischemia caused an increase in the levels of active caspase-3 and hyperphosphorylated tau, both of which were prevented by either NAP or caspase-inhibitor treatment. Our data suggest that, in this model system, caspase activation may be an upstream event to tau hyperphosphorylation, although additional studies will be required to fully elucidate the cascade of events.

1. Introduction

NAP is an 8 amino acid peptide, which was identified as an active neuroprotective fragment of activity-dependent neuroprotective protein (ADNP) [1]. NAP was found to be neuroprotective in various in vivo and in vitro models of neurodegeneration [2]. NAP treatment was shown to reduce two major pathological markers—tau hyperphosphorylation [3–5] and caspase-3 activation/apoptosis [6, 7]. Hyperphosphorylated and aggregated tau, originally detected in Alzheimer's disease (AD) brains by Grundke-Iqbal and colleagues [8], is a hallmark of a group of diseases, generally referred to as "tauopathies" which differ from each other by genetic background and by additional pathological and phenotypic characteristics [9].

Tau is a microtubule-associated protein (MAP) which promotes microtubule stabilization. The first study that reported disassembly of microtubules from AD brain due to the abnormal hyperphosphorylation of tau was by Iqbal et al. [10]. Hyperphosphorylated tau loses its microtubule affinity causing a change in microtubule dynamics towards disassembly [11–16] and further accumulation of aggregated tau. Alonso et al. originally showed that AD abnormal hyperphosphorylation of tau causes not only loss of function but also the gain of toxic function, with hyperphosphorylated tau blocking microtubule assembly in the presence of normal tau [17] and promoting the formation of normal tau containing tangles [18]. The most prevalent tauopathy is Alzheimer's disease (AD), and other tauopathies include a selection of frontotemporal dementia/degeneration, with pure tauopathies like progressive supranuclear palsy (PSP) [19].

One of the hallmarks of tauopathies is the accumulation of neurofibrillary tangles (NFTs). NFT accumulation correlates with the severity of dementia and memory loss [20–22] and with neuronal degeneration in AD and PSP [23, 24]. Recent studies associated the spread of tauopathies with propagation of prion-like protein inclusions [25] and suggested transsynaptic spread of tau [26]. However, several studies showed that soluble defective tau is also correlated

Table 1: Models of NAP protection against tau pathology or increased markers of apoptosis.

Models of tau pathology detecting protective effects when treated with NAP
(i) Transgenic ADNP heterozygous mouse [4]
(ii) Transgenic human double-mutant tau mouse [5]
(iii) Triple transgenic mice expressing the amyloid (Aβ) precursor protein APP(Swe), presenilin PS1 (M146V), and tau (P301L) [45, 46]
(iv) Mixed neuroglial primary cultures treated with Aβ (1–42, 2.5 μM) [3]
(v) Primary cultures of astrocytes [47]
Models of apoptosis detecting protective effects when treated with NAP
(i) A stroke model using spontaneously hypertensive rats which underwent permanent middle cerebral artery occlusion [48]
(ii) A rat model of diabetes (streptozocin toxicity) [7]
(iii) A rat model of epilepsy [49]
(iv) PC-12 cells exposed to H_2O_2 [50]
(v) Primary neuronal cultures subjected to ischemia/ reperfusion schedule of 3 h/3 h [39]

with cognitive deficits [27–29], causing synaptic loss and gliosis before NFTs formation [30]. Tau is also a substrate of multiple caspases, which cleave it and promote its pathologic aggregation [31–33]. Cleaved and hyperphosphorylated tau is found in deposits in AD brains, and it was found that truncated tau promotes apoptosis [34]. The relationship between tau hyperphosphorylation and cleavage is not completely understood. Though some evidence suggests that caspase cleavage of tau is not necessary for tau hyperphosphorylation, this question should be further clarified [35, 36]. Caspases are known to play a role in AD (for review, see [37]), and caspase effects on modifications of tau are of a great interest. Furthermore, treatment of an AD mouse model with a broad-spectrum caspase inhibitor was lately shown to reduce tau pathology but not amyloid-β (Aβ) pathology [38].

To date, we have tested NAP effects on either tau hyperphosphorylation or on caspase activation in separate models, showing inhibition of tau pathology or inhibition of caspase activation (or other apoptotic markers) (Table 1). The data emerging from these studies raise the question whether there is a dependency between the effects of NAP on the two pathologies. In the current study, we used an in vitro model of ischemia in which NAP treatment reduced apoptosis [39]. As animal models of ischemia exhibit tau hyperphosphorylation [40–42], we investigated whether the in vitro ischemia model mimics the in vivo situation. Thus, the aim of the current study was to identify a possible interaction between caspase activity and tau hyperphosphorylation, and to test the effect of NAP on both events under ischemic conditions.

2. Materials and Methods

All procedures involving animals were approved by the Animal Care Committee of Tel-Aviv University and further approved by the Israel Health Authorities.

2.1. Primary Neuronal Cultures. Primary neuronal cultures were produced from cortices obtained from 1-2-day-old Sprague-Dawley rat pups. Pups were decapitated, their skulls were removed, and their cortices were transferred to a Petri dish field with HBSS-HEPES buffer. Meninges were removed using tweezers under binocular. Neurons were produced with Worthington's papain dissociation kit (Worthington, cat.LK003150) according to the manufacturer's instructions. The cells were seeded in Neurobasal-A media (Invitrogen, cat. 10888022) fortified with 1% Glutamax supplement (Invitrogen, cat. 35050038) and 1% Neurocalm (Stemcells technologies, cat. 05711). All tests were executed 5-6 days in vitro (DIV).

2.2. Antibodies. The following antibodies were used for immunefluorescence: NeuN (Neuronal Nuclei, Chemicon, cat. MAB377), diluted 1 : 100, and GFAP (glial fibrillary acidic protein, Abcam, cat. 7260), diluted 1 : 500. The following antibodies were used for immunoblot analysis: p-tau^{202} (Anaspec, cat. 28017) 0.25 μg/mL, total tau (MBL international, cat. AT-5004) 0.5 μg/mL, and active caspase-3 (Abcam, cat. 2302) 1 μg/mL. Fluorophore and horseradish peroxidase conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc.

2.3. Analysis of Culture Purity. Cells were seeded in 24 well plates on poly-D lysine- (PDL-) coated coverslips (Sigma-Aldrich, cat. p-6407) at a density of $0.5 * 10^5$ cells/well. At 5–6 DIV, the cultures were fixed using a 4% paraformaldehyde (PFA) solution. Antigen retrieval, if needed, was performed by boiling the sample in sodium citrate buffer (pH = 6) for 20 minutes. Permeabilization was performed with a 0.2% Triton-X solution. Nonspecific antigen binding was blocked by incubation in a 2% bovine serum albumin (BSA) solution, then primary antibody (Ab) diluted in primary antibody diluent (Biotech applications, cat. MSBA-AbDil) was added for 1 hour at RT. Cells were washed three times in 2% solution of BSA and incubated in secondary antibody solution for 30 minutes in the dark. After an additional wash, PBS + DAPI (4′,6-diamidino-2-phenylindole, a fluorescent stain that binds strongly to A–T-rich regions in DNA, Invitrogen) was applied for 5 minutes, in order to stain cell nuclei. The coverslips were put on an antifading buffer on a carrier slides. For analysis of culture purity, we used 3 cultures produced independently. We used Zeiss fluorescence microscope and

camera to obtain 10 pictures (×20 magnification) from each culture. Each picture contained a minimum of 30 cells. NeuN-positive and GFAP-negative cells were considered neuronal and were expressed as % of the total DAPI count.

2.4. OGD Treatment. Cells were seeded on PDL-coated plates employing two different plating conditions (Corning): (1) 10 cm Petri dishes, $6 * 10^6$ cells per dish (for protein extraction), and (2) 48 well plates $0.5 * 10^5$ cells/well (for viability tests). After 5-6 DIV, the condition media (CM) was collected, filtered, and kept at 37°C. Cultures were washed twice with 37°C PBS, and the experimental media was added (PBS for the OGD groups, fresh media for the control group). Treatments were added to the experimental media (QVD-OPH, a broad-spectrum caspase inhibitor [43], 20 μM diluted in DMSO (Biovision, cat. no. 1170) or 10^{-5} M NAP diluted in PBS or diluents only). The culture dishes were placed in an ischemic chamber (Billups-Rothenberg, Inc., cat. MIC 101) which was filled with a $5\%CO_2/95\%N_2$ gas mixture and sealed, and the chamber was placed in an incubator for 2 hours; at 37°C. Two experimental modes were applied, in the first one (used for NAP protecting concentration curve), gassing was for 25 minutes followed by sealing for 2 hours, in the second paradigm (used for calibration of caspase inhibition and protein analysis), gassing was for 5 minutes only. O_2 percentage was monitored with O_2 meter (HUMIL International Corporation, cat. PO2-250) and did not exceed 1.2% any time during the OGD period. At the end of the OGD period, the culture dishes were removed from the chamber. For protein extraction, the experimental media was removed and protein was extracted as described below.

2.5. Viability Assay. Cells were seeded in 48 well plates at a density of $0.5 * 10^5$ cells/well (as described above). Following the OGD period, the condition media (CM) which was removed at the initiation of the OGD treatment (see above) was reapplied to the corresponding treatments. The MTS assay was used to assess viability (Promega, cat. G3580). Absorbance at 490 nm was measured in a SpectraMAX 190 plate reader, (Molecular Devices, Inc.). The results were analyzed using SoftMax Pro software version/year (Molecular Devices, Inc.) and expressed as % of the control. The results summarize 3 independent experiments with 5 replicates per treatment per experiment. Data are presented as means/medians ± SE.

2.6. Immunoblots. Immediately after OGD treatment (following gassing mode of 5 minutes of gassing and 2 hours OGD period), experimental media was discarded, and the dishes were washed twice with PBS. Protein was extracted using RIPA buffer with anti-proteases (ROCHE, cat. 11 873 580 001) and anti-phosphatases. Protein concentration was quantified using Pierce BCA protein assay reagents (Pierce, cat. 23223, 23224) with bovine serum albumin (BSA) as a standard protein. Equal amounts of protein diluted in sample buffer were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (12% gels or 4–20% gradient gels, nUView, cat. NG21-420). Proteins were transferred to nitrocellulose membranes using standard techniques, as before [44]. Membranes were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBSt) and then incubated overnight with primary antibody. Membranes were washed 3 times with TBSt and incubated with secondary Ab for 1 hour at RT. Pierce ELC substrate or Pierce super signal ECL was used to visualize proteins (cat. 32106, 34080). Signal density was detected with the DNR bioimaging systemsMiniBIS pro (DNR Bio-Imaging Systems Ltd.) and TotalLab TL-100 software (Nonlinear Dynamics Ltd.).

2.7. Statistical Analysis. SPSS 19.0 for windows (http://www-01.ibm.com/software/analytics/spss/) was used for statistical analysis. For viability assessment, 3 independent experiments were conducted with 5 trials per treatment in each experiment. Data were analyzed using a one-way ANOVA with 5% selected the level of significance, and post hoc Scheffe and LSD corrections. The effect of NAP was evaluated in 14 independent experiments, of which in 4 experiments QVD-OPH effect was also tested, and the levels of significance were evaluated using ANOVA with post hoc LSD.

3. Results

3.1. Characterization of Culture Purity. Culture purity was analyzed using immunofluorescent staining with cell-specific markers. After 5-6 DIV (days in vitro), 95.9 ± 0.96% (SEM) of the cells (counted using nuclear DAPI stain) were positive for the neuronal NeuN marker and negative for the astrocytic marker, GFAP. A representative immunefluorescence stain in Figure 1 shows neuronal cells stained with NeuN in red, and astrocytes stained with GFAP in green.

3.2. NAP Protective Effect Following 2 Hours of OGD. Cell viability was evaluated following a 25-minute gassing period and an additional 2 hours of OGD. The results were calculated and expressed as % of the control group (Figure 2). Under this paradigm, ∼70% cell death was observed. Treatment with NAP increased cell viability immediately following OGD ($F = 17.667$, df $= 7$, $P < 0.001$). Post hoc pairwise comparisons (LSD) indicated that NAP concentrations of 10^{-7} M and 10^{-5} M significantly protected against ischemia (Figure 2, $P \leq 0.05$).

3.3. Apoptosis Was the Main Type of Cell-Death Following 2 Hours of OGD. Using the broad spectrum caspase inhibitor QVD-OPH, we tested the extent of apoptotic death following 5-minute of gassing and 2 hours of OGD. Cell viability was evaluated and compared to the control group (Figure 3). Under this paradigm, ∼55% cell death was observed. Here, QVD-OPH ($2 * 10^{-5}$ M) treatment significantly increased cell viability to the control levels. In the same experiment, NAP in the same experiment, NAP (10^{-5} M) treatment rescued from the apoptotic cell death ($F = 16.090$, df $= 7$, $P < 0.001$). Post hoc pairwise comparisons did not detect any

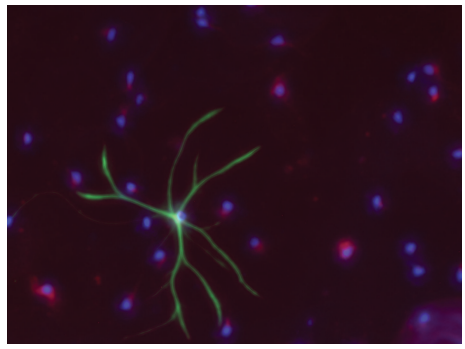

Figure 1: Culture purity: at 5-6 DIV, cells were fixed and immunofluorescence staining was performed using the astrocyte marker GFAP (green), the neuronal marker NeuN (red), and DAPI stain (blue) for nuclei. Quantification of neuronal cells was done using 10 random fields from each of 3 experiments (×20 magnification). 95.9 ± 0.96% of the total cells (DAPI stain) were recognized as neurons (NeuN positive, GFAP negative).

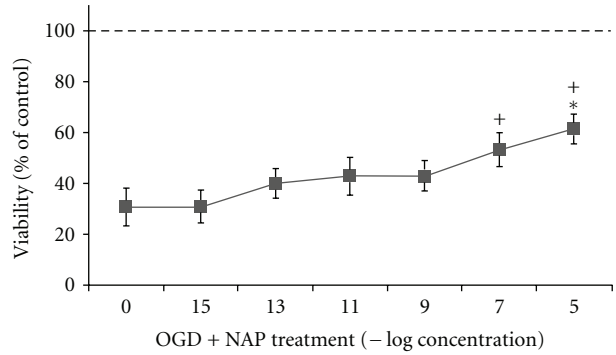

Figure 2: NAP protects from cell death in a dose dependent manner following 2 hours of OGD insult, but not after additional 24 hours reperfusion. Primary neuronal cultures (5-6DIV) were subjected to 2 h of ischemic insult. Cell viability was evaluated using MTS viability assay. Data was normalized to % of control. Results are shown as mean ± SE. *Significantly different from OGD with no NAP, OGD + NAP10^{-15} M, OGD + NAP 10^{-13} M, OGD+NAP 10^{-11} M (3 independent experiments are summarized, ANOVA, with post hoc Scheffe, significant difference considered to be $P \leq 0.05$). Using LSD post hoc, NAP 10^{-5} M (+), 10^{-7} M are significantly different from the OGD group ($P < 0.05$).

significant differences between the caspase inhibitor, NAP, and control no-ischemia groups.

3.4. Active Caspase-3 Expression Was Increased Following OGD and Reduced Due to NAP Treatment. The levels of active caspase-3 were evaluated immediately after the OGD period using immunoblots with a specific active caspase-3 antibody and further quantified and normalized to actin. As shown in a representative blot in Figure 4(a), OGD treatment increased active caspases-3 levels. This increase was prevented by NAP treatment (10^{-5} M) or QVD-OPH (caspase inhibitor) treatment ($2 * 10^{-5}$ M). Figure 4(b) depicts the level of active caspase-3 expressed as % of control. A significant increase in active caspase-3 expression was induced by OGD and partially but significantly prevented by NAP treatment ($F = 7.880$, df = 2, $P = 0.004$).

3.5. Tau Hyperphosphorylation Was Increased Following 2 Hours of OGD; P-Tau Increase Was Prevented by Either NAP Treatment or QVD-OPH Treatment. phospho-tau (p-tau) levels were detected by immunoblot, with a p-tau^{202}-specific antibody. A representative blot is shown in Figure 5(a). P-tau levels, were quantified, normalized to total tau levels and expressed as % of the p-tau levels in the control culture (Figure 5(b)). Phospho-tau levels were significantly increased following the OGD insult. Both NAP treatment (10^{-5} M) and QVD-OPH (caspases inhibitor) treatment ($2 * 10^{-5}$ M) prevented p-tau increase (ANOVA with post hoc LSD, $P \leq 0.05$), ($F = 5.633$, df $= 2, P = 0.012$).

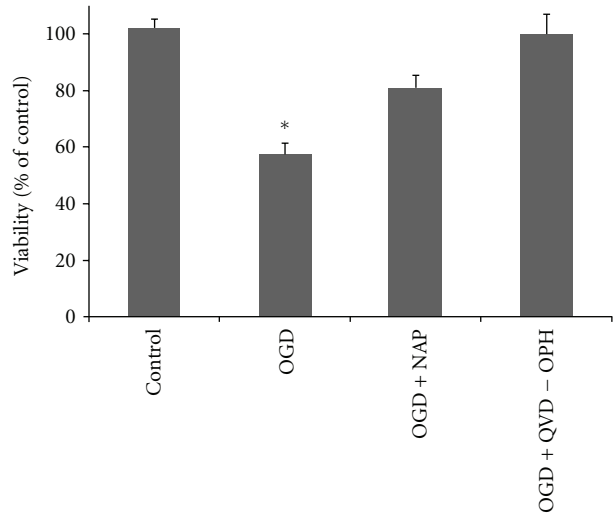

Figure 3: Following 2 hours of OGD, cells died exclusively from apoptosis. Cultures were treated with either NAP (10^{-5} M) or QVD-OPH ($2 * 10^{-5}$ M) and subjected to 2 h of ischemic insult. Cell viability was evaluated immediately following the ischemic period using MTS viability assay. Data was normalized to % of control. Results are shown as mean ± SE; *significantly different from all other groups, $P \leq 0.05$ (ANOVA, with post hoc Scheffe).

4. Discussion

Focusing on the protective properties of NAP, two central characteristics of neurodegeneration have been shown to be inhibited by NAP treatment both in vivo and in vitro: tau hyperphosphorylation [3–5] and apoptosis [6, 7, 51]. The main goal of the current study was to look for an association between these two pathological cascades. Our experimental setup used primary neuronal cultures (95.9 ± 0.96% purity), subjected to 2 hours of OGD with no reperfusion period. Under this paradigm, apoptosis was found to be responsible for all cell death in that it was inhibited by QVD-OPH. Our results showed that inhibition of caspase activity prevented tau hyperphosphorylation, leading us to conclude that in the current experimental

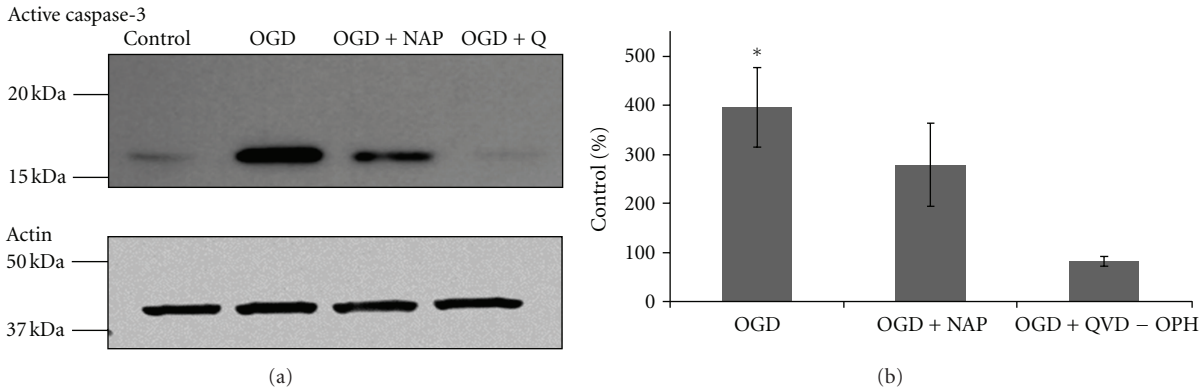

Figure 4: An increase in active caspase-3 levels induced by 2 hours of OGD was diminished by NAP treatment (10^{-5} M). Cultures were treated with 10^{-5} M NAP or with 20 μM QVD-OPH (broad spectrum caspase inhibitor) exposed to OGD insult for 2 hours. Proteins were extracted and analyzed using immunoblot with a specific antiactive caspase-3 antibody. A representative blot of active caspase-3 antibody is exhibited in (a). (ANOVA with post hoc LSD, $P \leq 0.05$) (b).

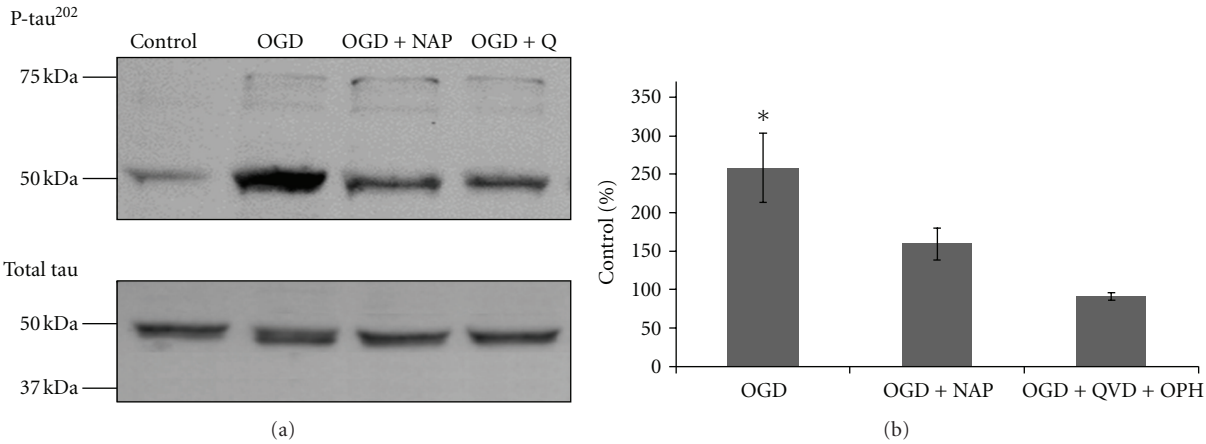

Figure 5: 2 hours of OGD caused an increase in p-tau^{202} levels, prevented by either NAP or QVD-OPH treatment. Cultures were treated with 10^{-5} M NAP or with 20 μM QVD-OPH (broad spectrum caspase inhibitor, indicated as OGD + Q) and exposed to OGD insult for 2 hours. Proteins were extracted and analyzed using immunoblot with a specific anti-p-tau^{202} and antitotal tau antibodies. A representative blot of p-tau^{202} and total tau is exhibited in (a). P-tau levels were quantified and normalized to total tau levels (ANOVA with post hoc LSD, $P \leq 0.05$) (b).

and cellular conditions, caspase activation is an upstream event to tau hyperphosphorylation. We further evaluated the effect of NAP on cell viability, caspase-3 activation, and tau hyperphosphorylation. Viability assays showed that NAP treatment rescued cells from apoptosis as demonstrated by the reduction in active caspases-3 following NAP treatment. NAP treatment also prevented tau hyperphosphorylation after the OGD. Combining the effect of NAP on active caspases-3 and tau hyperphosphorylation, it seems likely that caspases-3 or an upstream pathway is targeted by NAP activity in isolated neuronal cells that are metabolically stressed.

A careful look should be given to the concentrations by which NAP had a significant protective effect. Previous data, including ischemia-reperfusion experiments conducted in primary neuronal and neuroglial cultures, exhibited protection from cytochrome-c release, microtubule breakdown and reduction in MAP2 intensity using NAP concentrations of 10^{-15} M–10^{-8} M [39, 52]. Though most studies used femtomolar concentration of NAP, Pascual and Guerri [53] used concentration of 10^{-7} M to show NAP protection in a model neurons cocultured with astrocytes obtained from prenatal ethanol-exposed fetuses. In our model, protection was detected when treating with NAP at concentrations $\geq 10^{-7}$ M. The differences in the potent NAP concentration could be explained by the combination of the acute insult applied to a relatively pure culture of immature neurons. Immature neurons (2–4 DIV) in a pure neuronal culture (99% purity) were shown to go through spontaneous apoptotic death which was prevented when astrocyte condition media was added to the neuronal cultures. In comparison to neuroglial mixed culture, pure neuronal cultures were also found to be more sensitive to excitotoxic insult that also plays a role in the ischemia induced cell death [54]. We have previously shown that neuronal protection in the absence of glia required increased concentrations of NAP as

compared to mixed neuroglial cultures [55]. Furthermore, the original discovery of the NAP containing protein, ADNP, was as a glial protein providing neuroprotection. ADNP is essential for central nervous system development, expressed in specific brain tissue in the adult brain [1], and is also deregulated and may be overexpressed following acute brain damage [56] and in animal models of neurodegeneration [57, 58]. ADNP-like immunoreactivity is secreted from glial cells [59] further providing neuronal protection [4, 53, 60]. In this respect, NAP protected against ADNP deficiencies as outlined in the Introduction [4]. NAP was further shown to promote neurite outgrowth [61, 62] provide microtubule stabilization [63] and protect neurons in a broad spectrum of neuropathologies models [2]. Importantly, NAP also showed glial protection in vitro [64] and in vivo [7].

In summary, we demonstrated that NAP reduces caspase-3 activation, and tau hyperphosphorylation, suggesting that caspase-3 or upstream pathways may be targets for NAP activity. We believe these data provide additional insights regarding the molecular mechanism for NAP's neuroprotective activity and the intricate interaction between microtubules/tau and apoptotic mechanisms.

Disclosure

Professor I. Gozes serves as the Founder Scientist and Director at Allon Therapeutics Inc., the company that develops davunetide (NAP), (http://www.allontherapeutics.com/).

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Review Article

Structure and Pathology of Tau Protein in Alzheimer Disease

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Alzheimer's disease (AD) is the most common type of dementia. In connection with the global trend of prolonging human life and the increasing number of elderly in the population, the AD becomes one of the most serious health and socioeconomic problems of the present. Tau protein promotes assembly and stabilizes microtubules, which contributes to the proper function of neuron. Alterations in the amount or the structure of tau protein can affect its role as a stabilizer of microtubules as well as some of the processes in which it is implicated. The molecular mechanisms governing tau aggregation are mainly represented by several posttranslational modifications that alter its structure and conformational state. Hence, abnormal phosphorylation and truncation of tau protein have gained attention as key mechanisms that become tau protein in a pathological entity. Evidences about the clinicopathological significance of phosphorylated and truncated tau have been documented during the progression of AD as well as their capacity to exert cytotoxicity when expressed in cell and animal models. This paper describes the normal structure and function of tau protein and its major alterations during its pathological aggregation in AD.

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia characterized by memory impairment and alteration of diverse cognitive abilities. In association with the global trend of prolonging human life and increasing number of elderly in the human population, AD becomes one of the most important health and socioeconomic problems of the present. AD and related tauopathies are histopathologically characterized by slow and progressive neurodegeneration, which is associated mostly with intracellular accumulation of tau protein leading to the so-called neurofibrillary tangles (NFTs) and other inclusions containing modified tau [1]. Tau protein was discovered in the mid-1970s of the 20th century by studying factors necessary for microtubule formation. Tau protein promotes tubulin assembly into microtubules, one of the major components of the neuronal cytoskeleton that defines the normal morphology and provides structural support to the neurons [2]. Tubulin binding of tau is regulated by its phosphorylation state, which is regulated normally by coordinated action of kinases and phosphatases on tau molecule [3, 4]. In pathological conditions, such as the case in AD, not only does abnormal phosphorylation of tau protein decrease its tubulin binding capacity leading to microtubule disorganization, but also this protein self-polymerizes and aggregates in the form of NFTs [5, 6].

2. The Tau Gene

The human tau gene is located over 100 kb on the long arm of chromosome 17 at band position 17q21 and contains 16

exons. Exon 1 is part of the promoter and is transcribed but not translated. Exons 1, 4, 5, 7, 9, 11, 12, and 13 are constitutive exons. Exons 2, 3, and 10 are alternatively spliced and manifesting in the adult brain. Exon 2 can appear alone, but exon 3 never appears independently of exon 2 [7]. In the central nervous system, alternative splicing of exons 2, 3, and 10 results in the appearance of six tau isoforms that are differentially expressed during development of the brain [7].

3. Structure and Function of Tau Protein

Tau protein belongs to a group of proteins referred to as Microtubule-Associated Proteins (MAPs), that in common are heat resistant and limited affected by acid treatment without loss their function [8]. This property observed in tau is due to a very low content of secondary structure. In fact, a number of biophysical studies revealed that tau is a prototypical "natively unfolded" protein [9–11]. Since disordered proteins tend to be highly flexible and have variable conformations, they have not been amenable for structure analysis by crystallography so far. Thus nuclear magnetic resonance spectroscopy is the only plausible method that allows a description of their conformations and dynamics with high resolution [12]. Now it is possible to obtain the complete backbone assignment of 441-residue tau (the longest tau isoform found in the human central nervous system; Figure 1). This makes it possible to probe the structure and dynamics of the full-length soluble protein and determine the residues involved in the interaction between tau and microtubules at single residue resolution [13].

Six isoforms of tau protein differ according to the contents of three (3R) or four (4R) tubulin binding domains (repeats, R) of 31 or 32 amino acids in the C-terminal part of tau protein and one (1N), two (2N), or no inserts of 29 amino acids each in the N-terminal portion of the molecule. These isoforms, which vary in size from 352 to 441 amino acid residues, are related to the presence or absence of sequences encoded by exons 2, 3, or 10. Inclusion of the imperfect repeat region encoding exon 10 leads to the expression of tau containing four microtubule-binding repeats (MTBRs) (4R tau: 0N4R, 1N4R, 2N4R), while exclusion of exon 10 results in splicing products expressing tau with three MTBRs (3R tau: 0N3R, 1N3R, 2N3R) [7, 14]. These six isoforms are also referred to as τ3L, τ3S, τ3, τ4L, τ4S, and τ4 [15]. Primary sequence analysis demonstrates that tau consists of a half-N-terminal acidic portion followed by a proline-rich region and the C-terminal tail, which is the basic part of the protein. The polypeptide sequences encoded by exons 2 and 3 add acidity to tau, whereas exon 10 encodes a positively charged sequence that contributes to the basic character of tau protein. On the other hand, the N-terminal region has an isoelectric point (pI) of 3.8 followed by the proline-rich domain, which has a pI of 11.4. The C-terminal region is also positively charged with a pI of 10.8. In other words, tau protein is rather a dipole with two domains of opposite charge, which can be modulated by posttranslational modifications [16]. Because each of these isoforms has specific physiological roles, they

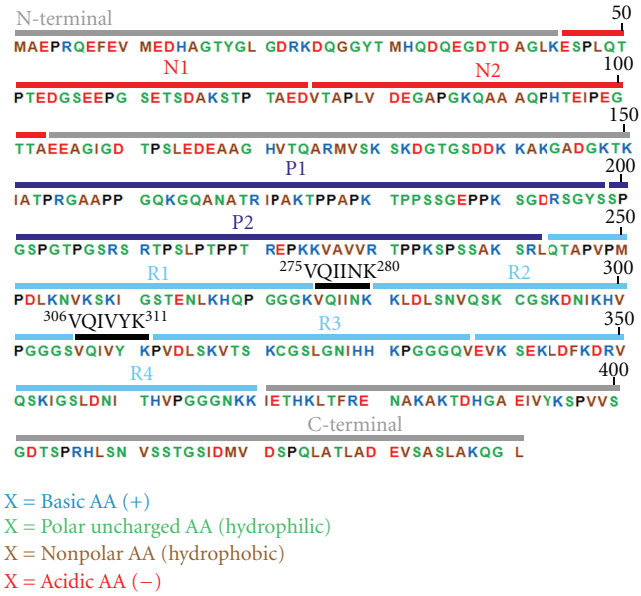

Figure 1: Amino acid sequence of the longest tau isoform (441 amino acids). N1 and N2: the polypeptide sequences encoded by exons 2 and 3; P1 and P2: proline-rich regions; R1–R4: microtubule-binding domains encoded by exons 9–12; ^{275}VQIINK^{280} and ^{306}VQIVYK^{311}: sequences with β-structure (modified by [13]).

are differentially expressed during the development of the brain. For instance, only one tau isoform, characterized by 3R and no N-terminal inserts, is present during fetal stages, while the isoforms with one or two N-terminal inserts and 3- or 4R are expressed during adulthood [7].

Tau protein is present in a greater extent in axons from neurons, but it also occurs in the oligodendrocytes. Another microtubule-binding protein referred to as MAP2 is located in the somatodendritic compartment of neurons, whereas MAP4 is much ubiquitous [17].

3.1. The Projection Domain and Its Interaction with Other Molecules. The two 29-amino-acid sequences encoded by exons 2 and 3 give different lengths to the N-terminal part of tau protein. The N-terminal part is referred to as the projection domain since it projects from the microtubule surface where it may interact with other cytoskeletal elements and the neuronal plasma membrane. In fact, the projection domains of tau protein determine spacing between microtubules in the axon and may increase the axonal diameter [7, 18]. Peripheral neurons often project a very long axon with a large diameter. This type of neurons contains an additional N-terminal tau sequence encoded by exon 4A and so generates a specific tau isoform called "big tau" [7, 18–20]. As to the interactions with other cytoskeletal components, tau protein binds to spectrin and actin filaments, which may allow tau-stabilized microtubules to interconnect with neurofilaments that restrict the flexibility of the microtubule lattices. Another molecule that interacts with tau protein is a peptidyl-prolyl cis/trans isomerase Pin 1. It isomerizes only phosphoserine/threonine-proline motifs and binds to

the tau protein after its phosphorylation on Thr^{231} residue. Isomerization induces conformational changes that make tau accessible for Protein Phosphatase (PP) 2A, which in turn leads to tau dephosphorylation. Protein Pin 1 regulates functions of tau protein and APP and is important for protection against the degeneration that occurs during the ageing process. Activity of Pin 1 is decreased by oxidation in AD [21]. Moreover, tau protein through its N-terminal projection domain may interact with intracellular membranous elements such as the mitochondria [22] and the neuronal plasma membrane [23]. In the cytosol of neurons the pools of tau protein in either phosphorylated or dephosphorylated forms are maintained in equilibrium by coordinated actions of kinases and phosphatases, respectively. Several studies in cell lines revealed that tau protein bound to the plasma membrane is dephosphorylated [24, 25]. Tau protein binds through its proline-rich region to the Src-homology 3 (SH3) domains of several proteins, including Fyn, a tyrosine kinase from the Src-family. The association of tau and Fyn depends on the phosphorylation state of tau, because insoluble PHF-tau isolated from AD brain does not bind to the Fyn SH3 domain [26]. Recently, Fyn has been demonstrated to play a role in protein trafficking [27]. For example, Fyn can increase the surface expression of the amyloid precursor protein (APP) through tyrosine phosphorylation [28]. The trafficking of tau protein to the plasma membrane is a bidirectional process, because increased tau phosphorylation induced by PP2A inhibition significantly reduces the proportion of membrane-associated tau. The active relocalization of tau in response to changes in phosphorylation suggests a possible role of this protein in intracellular signaling pathways [29, 30]. It was recently shown that tau binds to the Fyn in dendritic spines, and this interaction regulates N-methyl-D-aspartic acid (NMDA) receptor signaling [31]. Pathological tau may participate in the localization of Fyn kinase to the postsynaptic compartment, where it phosphorylates NMDAR subunits, causing increased inward Ca^{2+} conductance and leading to excitotoxicity [32]. *In vivo*, tau has been demonstrated to interact directly with ionotropic glutamate receptors [33]. In oligodendrocytes, the association of tau with Fyn regulates the outgrowth of cytoplasmic process [34]. Impaired interaction of Fyn kinase and hyperphosphorylated tau protein leads to hypomyelination and evolving demyelination of axons [34]. All these evidences indicate that the phosphorylated state of tau protein not only affects microtubule stability but also produces alterations on neuronal plasticity.

3.2. The Domain Associated with the Microtubules. Tau protein binds microtubules through some repeated domains (R1–R4) (encoded by exons 9–12) located at the C-terminus of the molecule (Figure 2) [35]. Each repeat consists of stretches of a highly conserved 18 residues that are imperfectly repeated three times in the fetal tau protein and four times in the adult specific form [35]. The repeats are separated from each other by 13- or 14-residue spacer regions. The main function of tau, aforementioned as a promoter of tubulin polymerization, depends mostly on the MTBR [35, 36]. It has been reported that *in vitro* tau

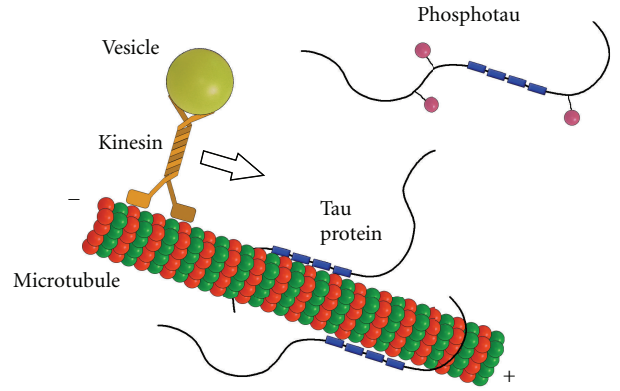

Figure 2: Normal function of tau protein. Tau protein stabilizes microtubules through four tubulin binding domains (blue boxes) in case of the longest isoform. Binding of tau protein to the microtubules is maintained in equilibrium by coordinated actions of kinases and phosphatases. The phosphorylation of tau (pink balls) regulates its activity to bind to microtubules and can affect axonal transport. Tau protein may inhibit the plus-end-directed transport of vesicles along microtubules by kinesin.

protein increases the rate of microtubule polymerization and concomitantly inhibits its rate of depolymerization [37]. The 18-amino-acid repeats bind to microtubules through a flexible array of distributed weak sites. The adult form of tau promotes assembly of microtubules more actively than fetal forms [14, 38]. Interestingly, the most potent part that induces microtubule polymerization is the interregion between repeats 1 and 2 (R1-R2 interregion) and more specifically the peptide ^{275}KVQIINKK^{280} within this sequence [7, 39]. This R1-R2 interregion is unique to 4R tau, adult specific, and responsible for the difference in the binding affinities between 3R and 4R tau [7, 35]. Recent evidence supports a role for the MTBR in the modulation of the phosphorylation state of tau protein. A direct and competitive binding has been demonstrated between this region (residues 224–236 according to the numbering of the longest isoform) and the microtubule on one hand and the same region with the PP2A on the other hand [40]. As a consequence, microtubules could inhibit PP2A activity by competing for binding to tau at the MTBR.

Microtubules contribute to diverse cellular processes such as cell morphogenesis, cell division, and intracellular trafficking [41, 42]. In cells, microtubules can change their lengths via dynamic instability [43]. They can serve as tracks for organelle transport mediated by microtubule-dependent motor proteins such as the plus-end-directed motor kinesin and its relatives, or the minus-end-directed motor dynein [44, 45]. These motors can transport their cargoes, for example, mitochondria [46, 47], lysosomes [48], peroxisomes [49], and endocytotic or exocytotic vesicles [50] towards the cell periphery or back towards the microtubule organizing center (MTOC), respectively. It has been shown that tau protein affects axonal transport [17, 51, 52]. Tau protein alters intracellular traffic due to its tight binding to microtubules and probably detaches the cargoes from kinesin. Nevertheless, tau protein has no influence on speed

of kinesin with cargoes [52]. This implies that the phosphorylation of tau should play an important role because this modification regulates tau's affinity to microtubules.

4. Tau Pathology

In AD, the normal role of tau protein is ineffective to keep the cytoskeleton well organized in the axonal process because this protein loses its capacity to bind to microtubules. This abnormal behavior is promoted by conformational changes and misfoldings in the normal structure of tau [53–55] that leads to its aberrant aggregation into fibrillary structures inside the neurons of demented individuals [56–58]. Thus, most of the altered pools of tau protein in the disease are redistributed and aggregated in both the somatodendritic compartment and isolated processes of affected neurons. Alterations in the amount or the structure of tau protein can affect stabilization of microtubules and other processes related to this protein [59, 60].

For instance, overexpression or mislocalization that increase intracellular concentration of tau may inhibit the plus-end-directed transport of vesicles along microtubules by kinesin so that the minus-end-directed transport by dynein becomes more dominant [17]. Inhibition of transport to the plus-end of microtubule slows down exocytosis and affects the distribution of mitochondria which become clustered near to the MTOC. The absence of mitochondria and endoplasmic reticulum in the peripheral regions of the axons could produce a decrease in glucose and lipid metabolism and ATP synthesis and loss of Ca^{2+} homeostasis [61] that leads to a distal degeneration process referred to as "dying back" of axons [62]. Moreover, phosphorylated tau protein has affinity to the kinesin and therefore is transported to the distal sites of neuropil. This may account for the observation that tangle pathology in AD appears to initiate distally and then spreads in a retrograde fashion to the perikaryon. This process may be a mechanism to protect the stability of the microtubules by transporting hyperphosphorylated tau more rapidly to other cellular locations where tau can form aggregates [51].

The mechanisms by which tau protein becomes a nonfunctional entity are in debate. Abnormal posttranslational modifications are proposed to be the main cause of this failure [63, 64]. In this regard, abnormal phosphorylation (hyperphosphorylation), acetylation, glycation, ubiquitination, nitration, proteolytic cleavage (truncation), conformational changes, and some other modifications [53, 65–73] have been proposed to cause the loss of normal function and the gain of pathological features of tau protein. In the upcoming sections we will focus our interest to describe evidence supporting abnormal phosphorylation, acetylation, and truncation of tau as major changes during the pathological processing of tau protein in AD.

4.1. The Hyperphosphorylation of Tau Protein. The phosphorylation of tau regulates its activity to bind to microtubules and stimulate their assembly as previously outlined. A normal level of phosphorylation is required for the optimal function of tau, whereas the hyperphosphorylated state makes tau to lose its biological activity. Regarding the potential propensity of tau protein to be phosphorylated, it was reported that the longest variant of tau protein (441 amino acid) holds about 80 potential serine or threonine phosphorylation sites [7]. Most of these potential sites are located at the vicinity of the MTBR in the proline-rich region and in the C-terminal extreme of the molecule of tau protein [16, 74] with the exception of Ser^{262}, Ser^{293}, Ser^{324}, and Ser^{356} (motif KXGS) in R1, R2, R3, and R4 domains [75, 76]. In the disease the abnormal phosphorylation of tau could be, but not mutually exclusive, the result of upregulation of tau kinase(s) or downregulation of tau phosphatase(s) [62, 74]. A number of these enzymes have been evaluated and those kinases that are believed to play the most important role in phosphorylation of tau in the brain include GSK-3β, cyclin-dependent kinase 5 (cdk5), cAMP-dependent protein kinase (PKA), and calcium/calmodulin-dependent kinase II (CaMK-II) [77]. GSK-3β may play major role in regulating tau phosphorylation in both physiological and pathological conditions. GSK-3β can phosphorylate tau on Ser^{199}, Thr^{231}, Ser^{396}, Ser^{400}, Ser^{404}, and Ser^{413} *in vivo* and *in vitro* (numbered according to the longest tau isoform), residues that are mostly phosphorylated in PHF-tau [78]. Aforementioned phosphorylation at Thr^{231} causes a local conformational change that allows the access of GSK-3β or other kinases to further phosphorylate tau. On the other hand, a complementary and opposite effect is for PP1, PP2A, PP2B, and PP2C that can dephosphorylate tau protein *in vitro* [79]. The activity of PP2A has been found to be reduced in selected areas of the brain of AD patients [4]. Overall tau phosphoprotein is at least three- to fourfold more hyperphosphorylated in the brain of AD patients than that in the brain of aged nondemented individuals [80].

At cellular level, abnormal phosphorylation of tau introduces alterations in several processes which are directly regulated by the suitable organization of the microtubule network. In a normal mature neuron, tubulin is present in over tenfold excess of tau, and thus practically all tau protein is microtubule bounded in the cell [81, 82]. In neurons affected in AD, abnormally phosphorylated cytosolic tau (AD P-tau) neither binds to tubulin nor promotes microtubule assembly [83–85]. Instead, this protein inhibits the assembly and disrupts the microtubule organization [83]. Moreover, it was reported that abnormally phosphorylated tau protein disengages normal tau from microtubules into the cytosolic phase [83], as much as 40% of the abnormally hyperphosphorylated tau in the brain of AD patients is present in the cytosol and not polymerized into paired helical filaments (PHFs) or forming NFTs [80]. The AD P-tau also removes the other two major neuronal MAPs, MAP1 and MAP2, from microtubule lattice [86]. This toxic feature of the AD P-tau appears to be solely due to its abnormal phosphorylation state because dephosphorylation of AD P-tau rescues this protein to perform its normal tasks [84].

By using a phosphorylation-dependent monoclonal antibodies against tau and mass spectrometry, it was reported that at least 39 phosphorylated sites in the tau molecule are associated with native PHF isolated from the brain of AD patients [87].

As to the *in situ* aggregation of hyperphosphorylated tau, a bunch of evidence has been generated over the years to identify abnormally phosphorylated tau as the major component of distinct neuropathological hallmarks that defines AD [6, 15, 65, 88–90]. Hyperphosphorylated tau has been observed as the major component of PHFs and straight filaments (SFs), NFTs, neuropil threads (NTs), and plaque-associated dystrophic neurites in the brain of AD cases [81, 91]. The density of NFTs distributed along the hippocampus, entorhinal cortex, and neocortex has been correlated with the degree of dementia in this disorder [92]. Moreover, the earliest accumulation of tau in the hippocampus of AD patients, prior to the formation of NFTs, has been viewed as a diffuse granular material immunoreactive to phosphorylation-dependent tau antibodies [93–95]. However, in the abnormal formation of PHFs, tau molecules may follow different alterations from which abnormal phosphorylation (although this may not be essential) causes misfolding and conformational changes that strength its abnormal aggregation [79, 96].

Recent studies demonstrated that hyperphosphorylation of tau occurs before its cleavage [97, 98] and that tau cleavage takes place before NFT formation [99]. In an *in vitro* model of ethanol-induced neuronal apoptosis, hyperphosphorylation of tau occurs before tau cleavage [98, 100]. Altogether, these results may indicate that abnormal phosphorylation is a key event that triggers the pathological aggregation of tau in AD.

4.2. The Acetylation of Tau Protein. The mechanism leading normal soluble tau to become hyperphosphorylated and disengaged from microtubules to form tau inclusions remains unknown and posttranslational modifications other than phosphorylation could regulate tau function and aggregation. Notably, reversible lysine acetylation has emerged as a potential regulatory modification implicated in AD and other neurodegenerative disorders. Recent studies demonstrate tau acetylation as a posttranslational modification that may regulate normal tau function [73, 101, 102]. Since acetylation neutralizes charges in the microtubule-binding domain, aberrant acetylation might interfere with the binding of tau to microtubule, leading to tau dysfunction, and suggests a role in pathological tau aggregation in AD and related tauopathies [73]. Increased tau acetylation on Lys^{280} could impair tau interactions with microtubules and provide increased pools of cytosolic tau available for pathological PHF aggregation [39, 101]. Consistent with this, Lys^{280}, located in the interrepeat region (^{275}VQIINKK^{280}), was identified previously as one of three lysine residues most critical in modulating tau-microtubule interactions [39]. Acetylation of tau aggregates was associated with hyperphosphorylated, ThS-positive tau inclusions in both Tg mouse models and human tauopathies [101]. This implies that negative regulation of tau function could occur via phosphorylation and acetylation events alone or in combination. The molecule of tau protein contains a lot of phosphorylation sites, as mentioned previously, and most of them occur in regions flanking the microtubule-binding repeat [74], in which Lys^{280} is located. Thus, tau hyperphosphorylation may render this residue available for subsequent acetylation, which would further impair microtubule binding and/or promote tau aggregation as well as further drive pathological alterations of tau. Although protein acetylation has been extensively studied in the context of histones and gene transcription, proteomics approaches have identified acetylated proteins in the cytoplasm and other organelles [103]. Recent study suggests that acetylation of Lys^{280} may be an intermediate step in tangle formation [102]. Acetylated Lys^{280} was mostly associated with intracellular neurofibrillary tangles compared to pretangles or extracellular ghost tangles throughout all Braak stages [73, 102]. Acetylated Lys^{280} also colocalizes with N- and C-terminal specific antitau epitopes. This indicates that it is present in neurofibrillary tangles prior to subsequent tau truncation [102].

Enzymes that add an acetyl group to the protein are called histone acetyltransferase (HAT) or lysine acetyltransferase. Of four major classes of HATs, p300/CBP (protein of 300 kDa and CREB-binding protein) and pCAF (p300-associated and CBP-associated factor) are exclusively present in metazoans [104]. Enzymes that remove an acetyl group from the protein are called histone deacetylase (HDAC) or lysine deacetylase. There are three classes of HDACs. The activities of HDACs in classes I and II (HADC1–11) depend on zinc as a cofactor; the activities of class III HDACs (sirtuins) depend on the relative levels of NAD+ and NADH [105, 106]. Of the seven members of mammalian sirtuins (SIRT1–7), SIRT1 is the most studied and is strongly implicated in aging-related diseases, including AD [107]. SIRT1 levels are reduced in AD brains, and the reduction correlates with the accumulation of hyperphosphorylated tau aggregates [108]. SIRT1 was found to reduce Aβ generation by activating transcription of a gene encoding α-secretase [109]. SIRT1 deficiency could also exacerbate the accumulation of Aβ, which could increase tau acetylation and tau phosphorylation even further. Since a decrease in SIRT1 activity can clearly have deleterious effects on neuron health, therapeutic strategies aiming at increasing sirtuins activity in AD brain warrant further research.

4.3. The Aggregation of Tau Protein In Vitro. The molecule of tau has long stretches of positively and negatively charged regions that are not conducive for intermolecular hydrophobic association [81, 110]. The β-structure in monomeric tau is concentrated only in R2 (exon 10) and R3 (exon 11), which can self-assemble by their own into filaments [111] and coassemble with heparin as an artificial inducer [112]. Evidence *in vitro* has revealed that self-aggregation of tau into filaments is inhibited by the presence of intact N- and C-termini, which lie down over the MTBR and avoid the interaction between these sticky domains [15]. Abnormal phosphorylation of the N-terminal and the C-terminal flanking regions may induce a relaxed structural conformation in the tau molecule that unclip both extremes from the MTBR region. This situation allows the self-interaction between these sticky domains in the formation of PHF/SF (Figure 3) [15].

Some other modifications such as deamidation could facilitate polymerization of tau protein. Curiously, several

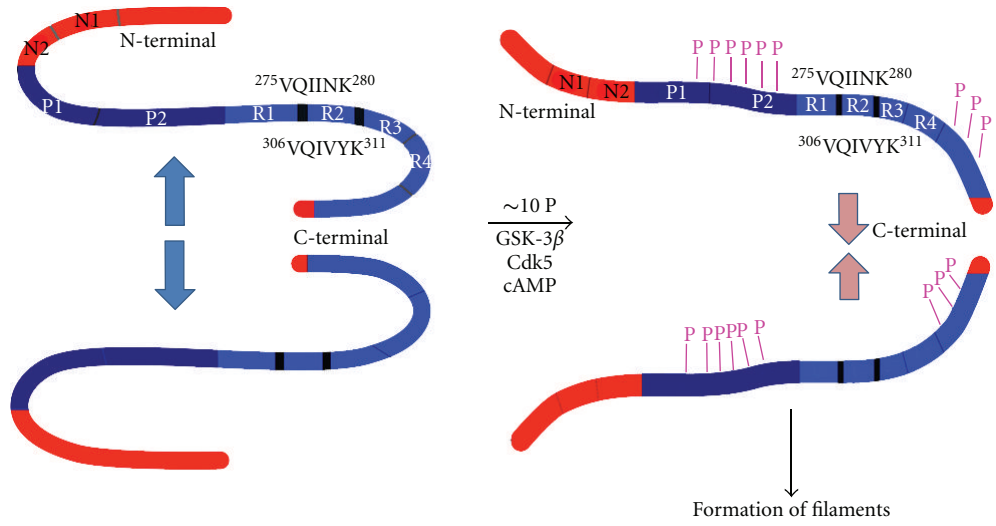

Figure 3: Phosphorylation of tau protein. Tau self-assembles mainly through the microtubule binding domains/repeat R3 in 3R tau proteins and through R3 and R2 in 4R tau proteins (R2 (^{275}VQIINK^{280}) and R3 (^{306}VQIVYK^{311}) have β-structure). N-terminal and C-terminal regions to the repeats are inhibitory. Hyperphosphorylation of tau neutralizes these basic inhibitory domains, enabling tau-tau interaction (phosphorylation sites indicated by violet Ps) (modified by [15]).

years later, it was shown that deamidation occurs in tau obtained from PHF [113]. Because a high concentration of tau protein is needed to polymerize [114], some suggest that other compounds, acting as cofactors, could be necessary to facilitate the self-assembly of tau protein [115–117]. Regardless of the phosphorylation state of tau protein, it was found that sulfoglycosaminoglycans (sGAGs), a class of polyanionic molecules, facilitate the polymerization of tau *in vitro* [115, 116]. Moreover, these sGAGs were found along with tau in NFTs, when the tau-neurofibrillary pathology was analyzed in the brain of AD cases [115, 116]. *In vitro*, tau polymerization paradigms also have utilized arachidonic acid as a polyanionic inducer [118], resulting in increased rates of filament formation. Other native polyanions such as the glutamic acid-rich region present at the C-terminal region of tubulin can also facilitate the aggregation of tau protein. This aggregation requires the presence of the third tubulin binding motif of the tau molecule [115]. Oxidation is another process that facilitates the aggregation of tau protein. Because 3R tau molecules contain only one cystein, oxidation of cystein produces disulfide cross-linking and thus self-assembly of tau protein [119]. It does not occur in 4R tau molecules with two cysteins, which may form intramolecular disulfide bonds [119].

Despite *in vitro* formed tau polymers have been demonstrated by spectroscopy, laser scattering, and electron microscopy [120–123], recent findings demonstrate that prefibrillar tau oligomers can be formed *in vitro* by light-induced cross-linking of tau with benzophenone-4-maleimide (B4M) [123]. These oligomers of tau were also observed *in situ* at the early stages of AD, when a monoclonal and specific antibody to these oligomeric entities of tau was assessed in the brain of AD cases [123]. Oligomeric species of tau protein are reported to have increased toxicity over soluble and high-ordered fibrillary aggregates such as NFTs [124–126]. In transgenic mice that overexpress tau, most of the observed cognitive alterations emerged at stages of profound occurrence of multimeric aggregates of tau and prior to the formation of NFTs [126].

4.4. The Truncation of Tau Protein. Proteolytic cleavage of tau protein, as an alternative mechanism involving in the abnormal aggregation of tau, was early proposed by Whischik's group at Cambridge University after extensive biochemical analysis of the minimal structure of the PHFs [69, 127, 128]. The minimal component of PHFs, referred to as the PHF core, was mostly composed of a fragment of tau only containing the region of the MTBR and ending at the position Glu^{391}. Until today, identification of the enzyme that produces this proteolytic cleavage is uncertain. However, the presence of this truncation associated with the neurofibrillary pathology has been demonstrated in the brain of AD patients [129, 130]. Furthermore, from *in vitro* paradigms of polymerization, tau constructs lacking the carboxy tail assembled much faster and to a greater extent than full length tau [131]. Despite these early evidences, attention was not focused for a while on the proteolysis of tau, and its contribution to the disease was uncertain. New findings show aberrant proteolysis in the brain of AD cases associated with programmed cell death [132, 133]. Further studies were dedicated to investigate the contribution of apoptosis and associated caspases into the neurodegenerative process underlying AD. In this regard, apoptotic cells were observed to proliferate in areas of the brain that were affected by fibrillary accumulation of tau protein and amyloid-β deposits [134–136]. Concomitantly, increased expression of several enzymes of the family of caspases was reported in the brain of AD cases [99, 137, 138].

Caspases are cysteine proteases that cleave aspartic acid residue in the canonical consensus sequence DXXD on the carboxy side of molecule. These enzymes participate in a proteolytic cascade leading to cell death via apoptosis. The major killer caspase in neurons is caspase 3 [139]. Members of the caspase family play a critical role in Aβ-induced neuronal apoptosis [140] and are activated in apoptotic neurons in AD [141]. It was known that tau protein contains several canonical sites for caspase cleavage [142, 143], from which a susceptible residue at Asp^{421} was reported to be cleaved *in vitro* by caspase 3 [72]. The cleavage at Asp^{421} released a discrete peptide (Ser^{422}-Leu^{441}) that is capable of forming an amphipathic α-helix [144]. Tau protein truncated at Asp^{421} assembled more readily than the full-length molecule [72, 144]. When a synthetic peptide comprising the fragment after caspase cleavage was added back to the tau molecule in a polymerization paradigm, assembly of this protein was inhibited.

In the disease, the occurrence of truncation of tau protein at Asp^{421} was corroborated in association with the neurofibrillary pathology by using the monoclonal antibody Tau-C3, which specifically recognizes this cleavage site generated by caspase 3 activity [72, 145]. Interestingly, phosphorylation of tau protein at residue Ser^{422} seemed to prevent the proteolytic cleavage of tau at Asp^{421} [146]. After truncation at Asp^{421} another cleavage of tau protein has been reported to occur at Glu^{391}. This state is recognized by antibody MN423, which indicates the transitions to "late" tangles [56, 67, 145]. Another truncation in the N-terminus of tau protein has been reported to occur at the residue Asp^{13}, which in this case is produced by caspase 6 activity [147]. Despite the *in vitro* demonstration that this truncation at the N-terminus is important to favor tau aggregation, its pathological meaning and occurrence in the brain of AD patients is still far from proven.

The pathological effect of C-terminus truncated tau over the normal functioning of the cells has been assessed in cultured cells and transgenic animal models. By using neuronal and nonneuronal cells, overexpression of truncated tau protein produces several alterations in the organization and functioning of membranous organelles, such as mitochondria and the endoplasmic reticulum. Even some examples of cell death by apoptotic mechanisms also have been reported [148–156]. In transgenics animals, truncated-tau carrying rodents have developed alterations in cognitive performance associated with neuronal death and abnormal aggregation of cleaved tau [100, 157–162].

Finally, the abnormal role of truncation of tau protein and its pathological significance in AD has been demonstrated by clinicopathological studies where the occurrence of truncated tau associated with fibrillary structures was analyzed during the development of the dementia [130, 145, 163]. These studies corroborate the importance of the truncated tau protein at both sites Asp^{421} and Glu^{391}. A positive correlation of these events with neuropathological progression of the disease was described by H. Braak and E. Braak [164] and a relationship to the clinical severity of dementia was demonstrated [130, 163]. Moreover, the presence of the Apolipoprotein-E (ε4) allelic variant was found in cases with an increased density of NFTs composed by the two variants of truncated tau [163].

In the hippocampus of AD patients, the maturation of NFTs is reported to be unsynchronized. Therefore these structures have different stages of tau processing [163]. It was reported that different populations of NFTs in the same hippocampal area were mutually exclusive when they were composed of either Asp^{421}- or Glu^{391}-truncated tau with no colocalization at any single point during the maturation of the NFTs [163]. During the progression of the disease, Asp^{421}-truncation is an early event that precedes the second truncation of the C-terminus at the Glu^{391}, the later occurring from intermediate to advanced stages of NFTs evolution [163]. A recent report indicates that tau protein in NFTs may be dually subjected to both apoptotic and proteosomal proteolysis since strong ubiquitination was found in Asp^{421}-truncated tau associated with the neurofibrillary pathology in AD [165].

By combining of antibodies that map different regions of the molecule of tau, a continuous and specific pathway of conformational changes and truncation of tau protein has been proposed to occur during the maturation of NFTs. These antibodies are, namely, conformational and phosphorylation-dependent and recognizing truncation sites [66, 67, 145].

These studies proposed that not only the number of NFTs but also the state of proteolysis of the C-terminus which is associated with conformational changes (structural modification along the tau molecule) defines the progression of AD [166]. All these findings together may support the relevance of truncation of tau protein as a pathogenic event and reliable marker for both diagnosis and therapeutic targeting in AD.

5. Conclusion

It is largely accepted that clinical manifestation of dementia in AD is due to the neuronal loss occurring in those areas of the brain associated with cognitive functions of the patients. Fibrillary inclusions are reported to be responsible for cell death. However, discrepancy has emerged from studies demonstrating that cognitive impairment in animal models occurs earlier than the initial formation of fibrillary structures. Extrapolation of these results to the real onset of the disease in humans is still considered inaccurate for some researchers. In this regard, a bunch of reports analyzing the brain of AD patients come to an agreement that fibrillary aggregation of tau is the best correlator with the onset and progression of dementia. It is mostly accepted that abnormal posttranslational modifications, that is, hyperphosphorylation, acetylation, glycation, nitration, truncation, and others, are responsible for altered tau structure in AD. Some of these events have been sequentially staged during the formation of NFTs and the evolution of the disease. Validation at clinicopathological levels with the load of abnormally phosphorylated and truncated tau has been demonstrated in populations of AD cases. Particularly abnormal phosphorylation, acetylation, and truncation are

further supported as pathological events by *in vitro* experiments demonstrating that these modifications increase fibrillization of tau and induce cell toxicity *in vitro*. Transgenic animals carrying these altered forms of tau protein also develop cognitive alterations. We believe that resolving the genesis of conformational changes of tau protein promoted by these posttranslational modifications and its role in fibrillization in disease are important achievements for assessing the potential of tau-directed therapies. Moreover, accurate determination of altered tau protein in the cerebrospinal fluid and other body fluids may provide better expectation to predict the onset and evolution of dementia.

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Review Article

Tau Phosphorylation by GSK3 in Different Conditions

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Almost a 20% of the residues of tau protein are phosphorylatable amino acids: serine, threonine, and tyrosine. In this paper we comment on the consequences for tau of being a phosphoprotein. We will focus on serine/threonine phosphorylation. It will be discussed that, depending on the modified residue in tau molecule, phosphorylation could be protective, in processes like hibernation, or toxic like in development of those diseases known as tauopathies, which are characterized by an hyperphosphorylation and aggregation of tau.

1. Introduction

Tau protein was discovered as one of the brain microtubule-associated proteins bound to in vitro assembled tubulin [1]. Tau protein appears as a series of different polypeptides on gel electrophoresis. These different forms are generated by alternative RNA splicing [2] or by different phosphorylation levels [3]. In every tau form, four different regions could be identified: the amino terminal region, the proline-rich region, the microtubule binding region, and the carboxy terminal region. Here, we will mainly comment on the tau molecule of the largest tau form present in the central nervous system (CNS) [4]. This molecule contains 441 residues. The N-terminal region (1–150 residues) and the proline-rich region (151–239 residues) have been involved in the interaction of tau protein with cell membranes [5, 6]. The microtubule-binding region (residues 240–367) contains four similar but not identical sequences involved in the interaction with tubulin [7], the main component of microtubules. The last part of the molecule is the C-terminal region (residues 368 to 441). Curiously, for a specific residue, threonine, there is a decreasing gradient in its presence from the N-terminal to the C-terminal region in human tau. Threonine is particularly abundant at the N-terminal region [7]. Some of these threonines, residues 17, 30, 39, 50, 52, or 95, present in human tau are not always conserved in other species and may have appeared during evolution in human tau. None of these human tau threonines have been found to be phosphorylated, but we cannot rule out that some of these sites could be modified with a very fast turnover.

2. Tau Functions

Tau is a neuronal microtubule-associated protein, and some of its functions are related to that association that may result in microtubule stabilization or the regulation of axonal transport, but tau protein is a "sticky" protein that could bind to other proteins, apart from tubulin (the main component of microtubules), which could also facilitate its subcellular localization at the cytoplasm close to the membrane or in the nucleus. On the other hand, in the neuron, tau is mainly present in axons, owing to a sorting mechanism in which its level of phosphorylation may play a role [8], but tau can be also localized, at a low level, to dendrites [9] where it binds to PSD95 protein, a postsynaptic component present in dendritic spines, which is bound to glutamate (NMDA) receptors [10].

Some of tau functions could be regulated by phosphorylation. In many cases, tau phosphorylation can cause its detachment from microtubules [11], and those functions like

microtubule stabilization and regulation of axonal transport could be affected by the change in the association of tau to microtubules.

3. Phosphotau

Tau is a phosphoprotein that could be modified by different protein kinases at tyrosine, threonine, or serine residues. In the longest CNS tau molecule, there are 80 serine/threonine residues that are mainly present at the proline-rich and C-terminal regions [11].

Tau kinases have been divided in proline- (PDPK) and nonproline- (NPDPK) directed protein kinases, being GSK3 the PDPK that could modify more sites in tau molecule [12]. These sites are also mainly present at the proline-rich and C-terminal regions.

GSK3 is a protein kinase originally identified due to its role in glycogen metabolism regulation. GSK3β, one of its isoforms, is abundant in the central nervous system and can modify several neuronal proteins like tau [13].

Tau hyperphosphorylation takes place, at the CNS, in several diseases (tauopathies), being the most predominant of these disorders, Alzheimer disease. In the tauopathies, tau protein is not only phosphorylated but also could be found in aggregated form. In Alzheimer's disease (AD) tau protein can be assembled into the paired helical filaments that form the aberrant structure known as neurofibrillary tangles [11]. Also, in AD, there is another aberrant structure the senile plaques, composed by the beta-amyloid peptide. A relationship between NFT and SP has been suggested through GSK3 (see below and [14]).

4. GSK3 and AD

In Alzheimer's disease postmortem brain material, tau phosphorylation has been found to increase at several sites modified by GSK3. In this way, the analysis of how an increase in GSK3 activity could modify tau protein has been deeply studied. It is known the involvement of factors like wnt, insulin, or insulin-like growth factor in the regulation of GSK3 [15]. It is also known that the accumulation of beta-amyloid peptide facilitates tau phosphorylation by GSK3 through the interference with insulin or wnt pathways [16, 17]. Also, mutations in the protein presenilin-1 (PS-1) that could result in the appearance of dementia with familiar origin may affect the interaction of PS-1 with the regulatory subunit of PI3 kinase, decreasing the activity of this kinase and the further activation of GSK3 [18]. On the other hand, in nonfamilial dementia like sporadic Alzheimer disease, the presence of apolipoprotein E4 isoform could facilitate a higher GSK3 activity [19]. Also, a GSK3 polymorphism has been associated with Alzheimer's disease [20], and an increase in GSK3 activity has been suggested to take place in the brain of AD patients [21]. Finally, GSK3 phosphorylates the majority of sites, on tau molecule, which are abnormally phosphorylated in AD [12].

One of the consequences of tau phosphorylation by GSK3 is a decrease of its interaction with microtubules [22], which could result in a destabilization of microtubule network [23]. Also, phosphorylation of tau by GSK3 (or other kinases) may result in an increase or a decrease in its interaction with other proteins. An increase was observed in its interaction with prolyl isomerase 1 (PIN-1), a chaperone protein that binds to phosphotau [24]. In this interaction phosphothreonine 231 is involved. On the other hand, Pin-1 could inhibit GSK3β, and a consequence of that is a reduced phosphorylation of amyloid precursor protein (APP), another important protein in AD. In the absence of phosphorylation, the turnover of APP is increased [25]. Also, Pin-1 acts on T668P motif in APP to promote non-amyloidogenic APP processing [26]. About GSK3 and APP processing it should be indicated a report indicating that GSK3, mainly GSK3α, can regulate APP processing and the production of amyloid beta peptide [27].

Going back to tau phosphorylation, it has been observed that the interaction of tau with cell membranes decreases upon its phosphorylation [6]. In this binding, phosphorylation of serine 202 may play a role. Thus, the balance of kinase and phosphatases acting on tau could be important for tau functions and dysfunctions.

5. Is Phosphotau Toxic or Protective in a Neuron?

Since tau phosphorylation may affect tau functions, it was suggested that phosphotau could be toxic. However, this point has been widely discussed and is commented if phosphorylated tau is toxic, protective, or none of the above [28].

Working on *Drosophila* as a model it was indicated that phosphotau is not toxic [29, 30]. Also, there are some works suggesting that tau toxicity could be dependent in an increase in its expression [31] or that the toxicity could be related to its fragmentation, yielding some specific peptides [32], or to its aggregation, but this point is also discussed, since some works suggest a protective role for large tau aggregates, although it may be insufficient in damaged neurons [33, 34]. More recently, it has been suggested a toxicity for tau oligomers [35]. A possible relation between tau phosphorylation and aggregation has been suggested. In a mouse model for temporal dementia, chronic lithium (a GSK3 inhibitor) treatment results in a decreased tau phosphorylation and aggregation [36]. In another study a similar result was found [37], being the site recognized by antibody PHF-1 one of the phosphosites with a higher decrease upon treatment with a GSK3 inhibitor. It may suggest that tau phosphorylation at some sites may facilitate tau aggregation. These results support the previous works indicating that large tau aggregates exert negligible neurotoxicity compared with soluble tau [38, 39].

A protective role for tau phosphorylation could be suggested when that modification could take place at threonine 181 [40] that could facilitate its binding to exosomes and the release of tau excess [41]. Also phosphorylation at serine 202 could prevent the tau proteolysis by calpain [42], or in some processes, like hibernation (see below), it could be also protective [43]. Although poorly understood, there is

an endocrine regulation of hibernation, and a recent study suggests that central system neurons are protected from low-temperature cell death by certain endogenous substances such as adenosine, opioids, and histamine [44].

On the other hand, tau toxicity resulting from tau phosphorylation could affect to axonal transport, mitochondrial respiration, or cytoskeletal changes [45, 46]. Also, the presence of hyperphosphorylated tau in dendritic spines impairs synaptic activity [47], and tau phosphorylation by GSK3 in the hippocampus results in a toxic gain of function [48, 49].

A way to study the role of tau phosphorylation on cell toxicity is the use of pseudophosphorylated tau variants where a serine or threonine residue is replaced by glutamatic acid. Thus, it has been found that pseudophosphorylation at the site recognized by antibody AT8, but not at the site recognized by abPHF-1, prevents the interaction of tau with neuronal membranes [50, 51].

Pseudophosphorylation of tau in ten different sites of a single molecule (including those sites recognized by AT8, ab PHF-1, and phosphothreonine 231) results in a toxic action and the induction of apoptosis [52].

Combined pseudophosphorylation at threonine 212, threonine 231, and serine 262 causes neurodegeneration, mainly through the presence of pseudophosphorylation at threonine 212 [53]. A possible mechanism has been involved for this toxicity: abnormal tau phosphorylation can result in the sequestration of unmodified tau [54] or in that of other microtubule associated proteins like MAP1 or MAP2 [23], promoting a net microtubule disassembly that could affect neuron cytoskeleton and promote neurodegeneration. On the other hand, tau phosphorylated at Thr 231 is used as a marker for neurodegeneration. It may suggest that tau modified at that residue could be toxic [55].

All those results suggest that, depending on the modified site, a protective or toxic function may take place and that it could be due to different conformational changes of tau molecule. Although tau is an unstructured molecule, it has been suggested that, in some conditions, it could have a paperclip conformation. In this conformation, the C-terminal end of tau interacts with the microtubule-binding domain, whereas the N-terminal end could bind to the C-terminal region, adopting a paperclip folding [56]. Pseudophosphorylation at the AT8 site makes the N-terminal region away from the C-terminal. On the other hand, pseudophosphorylation at the PHF-1 site decreases the interaction of C-terminal region with microtubule binding domain [57]. Thus, phosphorylation at different sites of tau molecule could have different consequences for tau conformations and function. In this way, we will comment more on the phosphorylation of tau on those sites recognized by ab AT8 or ab PHF-1.

6. The Progression of Alzheimer and the Phosphorylation of Tau by GSK3 at Two Different Sites

At the level of degeneration of a single neuron, occurring in Alzheimer's disease, it was suggested that first takes place phosphorylation of tau molecule at some specific sites and afterwards there is a further phosphorylation and aggregation of tau protein. Thus, early tau phosphorylation can be used as an initial marker for neurodegeneration [58].

The phosphorylation of tau, by GSK3, at specific sites can be analyzed by the use of antibodies that specifically recognize some of those sites. In this way, analysis of phosphorylation at serine 202 or at serines 396–404 could be achieved by using the antibodies AT8 [59] or PHF-1 [60], respectively. The uses of these antibodies have indicated that different tau molecules could be modified at those different sites [61]. On the other hand, in mature neurons tau phosphorylated at the site recognized by PHF-1 is preferentially present in the axon, whereas tau modified at AT8 site is located in the somatodendritic compartment [61]. Temporal lobe sections, including the entorhinal region and hippocampus, were studied using the antibody AT8 in AD patients and controls, to follow the consequences of that phosphorylation at the level of a single neuron and its possible relation with tau aggregation. The analysis showed that tau was modified at the site recognized by ab AT8 much earlier than the appearance of aggregated (fibrillar) tau, suggesting that phosphorylation at the AT8 site represents an earlier change than tau aggregation [58]. More recently, by looking at specific neurons in the brain of Alzheimer's disease patients like those present in the CA3 region of the hippocampus, the sequence for cellular changes related to tau phosphorylation and the posterior formation of aggregates has been studied with tau antibody AT8. This antibody recognizes the first stages of tau phosphorylation (pretangle stages) [62], whereas the reaction with tau antibody PHF-1 could correlate further phosphorylation steps and the formation of tau aggregates (tangle stages) [63].

Tau phosphorylation (recognized by AT8 antibody) at regions like CA3 of the hippocampus has been correlated with regression of synaptic components and memory deficits [64]. Indeed, although in AD there is neuron death, it has been suggested that there also could be alive neurons undergoing atrophy [65]. This atrophy could be the consequence of a metabolic decline [66] (that could be a very early sign of AD). Indeed, there is a reduced metabolism in those regions of the brain that are later affected in AD patients, and this glucose hypometabolism may reflect a reduced synaptic activity. This reduced metabolism may induce hypothermia, decreasing the activity of phosphatases that could result in the net phosphorylation of GSK3 substrates like tau protein [66].

A main difference between neuron atrophy and neuron death is that in the first case the neuron remains alive and could be possible to reactivate it. In fact, we have recently observed that in patients with AD the accumulation of phosphotau in a pretangle state does not induce changes in the dendrites of pyramidal neurons, whereas the presence of tau aggregates forming intraneuronal neurofibrillary tangles gives rise to a progressive dendritic atrophy and the loss of dendritic spines (synaptic disconnection) (Merino-Serrais P et al. in preparation). Thus, the presence of phosphotau in a pretangle state in neurons of the human brain does not necessarily result in severe and irreversible effects. Thus,

it is of great interest to search for possible physiological conditions in which neurons with a reduced metabolism and altered synaptic connectivity that may result in the lack of cognitive functions could be reactivated to regular functionally neurons. The group of Arendt et al. [67] answers to that question by showing that topor in hibernating animals shows a significant analogy with the pretangle stages of AD. Hibernation is a behavioral strategy used by some mammals to minimize energy expenditure under inhospitable environmental conditions. During hibernation overall metabolic rates are greatly reduced and neurons like those of the CA3 hippocampal regions have a reduced metabolism [68]. Moreover, tau phosphorylation also takes place in those neurons, at sites recognized by AT8 antibody, phosphorylation which is fully reversed in a few hours when these animals spontaneously interrupt torpor by an arousal leading the animals to euthermia for 24 h approximately [69]. Furthermore, it has been shown that there is a re-establishment of mossy fiber synapses in CA3 neurons after arousal [70]. Previous studies have shown that neuronal cell bodies, dendrites, and spines in hibernating ground squirrels retract on entry into torpor state. When they return to euthermia, there is a recovery of the arbor complexity and spine density [71].

Thus, if topor stage, in hibernation, is similar to the pretangle stages in AD and it could be reversed, we could ask if that reversion could also take place in AD. This question has pros and cons. One of the cons is that the primary structure of tau from hibernating animals could be different than that of a human being. Thus, tau from hibernating mammals have been sequenced [70], but no dramatic differences with human tau were found. Then, how can we increase metabolic rate in AD neurons? In a further analysis, tau protein from *Mesocricetus auratus* (another hibernating rodent) has been sequenced and characterized (Leon G et al. in preparation). The reason of that characterization has been to perform a first step to know if tau protein in hibernating animals, like *M. auratus*, could be taken as a single marker of the process or it may play a role in it. Thus, tau differences between this hibernating animal and nonhibernating organisms like the human being should be carried out. Our results have indicated the presence of several variations at the N-terminal half between *M. auratus* and human tau. Some of these variations have been also found in nonhibernating rodents. However, there are three variations, in that region, which are only present in the two hibernating rodents, *M. auratus* and *S. citellus*. In addition, there are another differences between *M. auratus* and human tau like the presence of 10 phosphorylatable residues present in human but not in *M. auratus* tau (Leon G et al. in preparation). Many of these phosphorylatable residues could be possible substrates for ck1, a protein kinase that has been related with circadian rhythm in nonhibernating organism [72, 73]. Although additional work should be done, our observations are compatible with a possible role of tau in the hibernation process and not being only found as a single marker.

It could be a possible relationship between hibernation/wake in hibernating mammals and sleep/wake in humans. Indeed, AD patients exhibit sleep/wake disorders [74]. However, sleep deprivation has been associated with GSK3 activation [75], whereas a net GSK3 phosphorylation takes place in hibernating mammals [76]. Nevertheless, modulators of sleep/wake cycle, like melatonin, have been suggested as possible therapeutical compounds for AD [77].

A more related behavior to that of hibernation could be anesthesia. Anesthesia leads to tau hyperphosphorylation through inhibition of phosphatase activity by hypothermia [78], a process that could be similar to that of hibernation. However, little is known about how is the recovery from phosphotau to unmodified tau after anesthesia. In addition, we do not know if tau phosphorylation is sometimes associated with the physiological conditions following hibernation or anesthesia or, like in AD, may play a toxic function [49]. Thus, if phosphotau could be toxic and it is difficult to active phosphatases for its dephosphorylation, the use of possible GSK3 inhibitors should be taken into consideration for the treatment of the disease.

Furthermore, we should look for better analysis to detect a decreased metabolic rate in damaged CA3 neurons in AD, and we should look for an answer about how to increase that metabolic rate in AD neurons. A probable common link among some of these disorders is the appearance of oxidative damage that results in neurodegeneration [79, 80].

A possible mechanism has been suggested to explain an energy depletion in AD. It involves that oxidative stress may lead to the formation of reactive carbonyl compounds [81] that appear to increase in AD patients. These compounds may decrease glucose metabolism yielding to energy depletion in neuronal cells [82]. Also, intracellular Aβ may cause mitochondrial defects and ATP depletion.

In summary, since neuron atrophy in neurodegenerative diseases, like AD, has been correlated with tau phosphorylation by GSK3, it should be tested in the future if by decreasing that phosphorylation that atrophy could be reversed or not.

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